Mechanisms of Activation and Inactivation of Rad53 Checkpoint Kinase

Thesis

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MECHANISMS OF ACTIVATION AND INACTIVATION OF Rad53 CHECKPOINT KINASE

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LIST OF ABBREVIATIONS

2D-gel-2 Dimensional Agarose Gel Electrophoresis

Mabs Monoclonal Antibodies

BSA-Bovine Serum Albumin

DSB-Double Strand Break

DSBR-Double Strand Break Repair

EDTA-Ethylen Di-ammino Tetraacetic Acid

GFP-Green Fluorescent Protein

HR-Homologous Recombination

NHEJ-Non Homologous End Joining

ORF-Open Reading Frame

RT-Room Temperature

SDS-PAGE-SDS-Poly-Acrilamide Gel Electrophoresis

SDS-Sodium Dodecyl Sulphate

SSA-Single Strand Annealing

ARS-Active replicatine sequence

SSC-Sodium Chloride Sodium Citrate

ssDNA-Single Stranded DNA

TBE-Tris Borate EDTA

TE-Tris EDTA

YPD-Yeast extract, Peptone, Dextrose

ABSTRACT

In many protein kinases, the phosphorylation of one or more residues in the activation segment is critical for driving the conformational change, which allows the activation of the kinase. The yeast checkpoint kinase Rad53 presents two phosphorylatable Threonine residues (T354 and T358) in the activation segment. In this thesis I have generated *rad53* mutants with affected activation segment functions resulting from Alanine or Aspartate substitution of single or both the T354 and T358. These results pinpointed functional regulatory roles of the phosphorylations of both Threonine residues in the catalytic activation of Rad53. Interestingly, the *rad53-T354D-T358D* mutation caused the accumulation of the S129-phosphorylated isoform of the histone H2A even during an unperturbed cell cycle, thus indicating the accumulation of spontaneous DNA lesions. We further found that the accumulation of DNA lesions in *rad53-T354D-T358D* cells is due to high abundance of Sml1, that is the physiological inhibitor of the Ribonucleotide Reductase, thus suggesting that the main source of the DNA lesions in *rad53-T354D-T358D* cells is an inadequate pool of the DNA replication precursors dNTPs. In this thesis I also present the production and characterization of novel monoclonal antibodies directed against various isoforms of Rad53, including antibodies directed specifically to autophosphorylated and active Rad53.

In conclusion, the results and reagents described in this thesis may help to elucidate the essential role of the checkpoint kinase Rad53 in preserving genome integrity.

INTRODUCTION

The checkpoints

The cell division cycle of eukaryotes consists of four different phases, G1, S, G2, and M, in which cells are preparing or actively involved in replicating (S phase) or segregating (M phase) their chromosomes. The timely faithful replication and segregation of genetic material is essential for cell survival and is ensured by surveillance mechanisms, called checkpoints. The term checkpoints is generally used to indicate the biochemical pathways which are activated in response to genotoxic agents, cell cycle blocks or alteration of particular cellular structures, even though surveillance mechanisms act both under normal and perturbed conditions (Amon, 1999; Elledge, 1996; Paulovich et al., 1997; Weinert, 1998). The first indication that cell cycle arrest caused by DNA damage is due to genetically controlled mechanisms and not to the damage itself came from studies carried out in *Escherichia Coli*, were it was found that mutations in certain genes relieved the septation block caused by DNA damage (Vinella and D'Ari, 1995). Later it was shown that cells from human patients suffering the genetic disorder Ataxia Telangiectasia (AT) were defective in slowing down DNA synthesis and mitosis in response to radiation treatments (Shiloh, 1997), suggesting that AT cells were unable to control cell cycle progression in response to DNA damage. The discovery that the *RAD9* gene was required to properly coordinate cell cycle transitions and DNA damage response in *S. cerevisiae* (Weinert and Hartwell, 1988) represented the starting point for a series of studies on the identification and characterization of checkpoint genes. It turned out that most of the proteins involved in checkpoints pathways are conserved throughout evolution underlaying their essential role in cell life (Melo and Toczyski, 2002). In table 1 the major players in checkpoint response in *S. cerevisiae* are indicated with the corresponding hortologue (from Harrison and Haber

2006).

	Budding yeast	Fission yeast	Human
PIKK	Mec1	Rad3	ATR
PIKK	Tel1	Tel1	ATM
Adaptor	Rad9	Crb2	53BP1, MDC1, BRCA1?
Rfc1 homolog	Rad24	Rad17	Rad17
9-1-1 clamp	Rad17	Rad9	Rad9
	Mec3	Hus1	Hus1
	Ddc1	Rad1	Rad1
MRX complex	Mre11	Mre11	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
BRCT domain adaptor?	Dpb11	Rad4/Cut5	TopBP1
Signaling kinase	Rad53	Cds1	Chk2
Signaling kinase	Chk1	Chk1	Chk1
Polo kinase	Cdc5	Plo1	Plk1
Securin	Pds1	Cut2	Securin
Separase	Esp1	Cut1	Separase
APC-targeting subunit	Cdc20	Slp1	$p55^{CDC}$/CDC20

Table1. List of checkpoint proteins in budding yeast with corresponding human and *S. pombe* hortologues.

The central player of the checkpoint response in *Saccharomyces cerevisiae* is the phosphatidylinositol 3' kinase-like (PIKK), Mec1. Mec1 is part of a sensor mechanism that detects DNA damage in the form of single stranded DNA (ssDNA) and transmits the checkpoint signal to a pair of transducing kinases, Rad53 and Chk1. These kinases amplify the signal and regulate the cell cycle machinery to allow checkpoint arrest prior to mitosis. In budding yeast the checkpoint arrest occurs prior to anaphase (in G2/M), with the sister centromeres of replicated chromosomes attached to mitotic spindle and under tension. Anaphase is inhibited preventing separase enzyme from cleaving cohesins and releasing the sister chromatids into anaphase, but there are likely to be other restrains as well. In mammalian cells, DNA damage during interphase prevents the accumulation of mitotic CDK1 activity and triggers a checkpoint-mediated arrest in G2, before nuclear envelope breakdown and chromosome condensation. In cells with repairable DNA damage, the checkpoint arrest is maintained until repair is completed but is then relieved allowing cells to re-enter into the cell-cycle. Studies of cancer predisposition syndromes and sporadic tumors in humans have identified mutations in many DNA damage checkpoint genes, underscoring the importance of the checkpoint response (Shiloh, 2003).

Triggering the DNA damage checkpoint

All aspects of DNA damage checkpoint in eukaryotes depend on members of the PIKK family, most famously human ATM and ATR (Abraham, 2001). ATM (Ataxia Telangiectasia Mutated) was identified as the gene mutated in the inherited cancer predisposition syndrome Ataxia telangiectasia (A-T), and is a key player in the response to double strand chromosomal breaks (DSBs) (Abraham, 2001). ATR (Ataxia Telangiectasia mutated and Rad3-related) is also a critical component of the checkpoint pathways, especially in response to lesions generating ssDNA (Abraham, 2001). In budding yeast, Mec1 and Tel1 are the counterparts of ATR and ATM respectively. The recruitment of checkpoint PIKKs at DNA lesions is considered the most upstream event that triggers checkpoint activation and cell cycle arrest. The creation of a system in which was possible to introduce a single DSB at a specific location helped in eliciting the complicated network reported in figure I. Much evidence suggests that the molecular specie recognized by the Mec1-Ddc2 complex is ssDNA. ssDNA is a common checkpoint signal that is formed during nucleotide and base excision repair, and at stalled replication forks (Carr, 2002; Sogo et al., 2002). This idea was already presented a decade ago in the context of the S-phase checkpoint in *Xenopus laevis* egg extract (Kornbluth et al., 1992), and recent work has further confirmed the view that ssDNA recruits Mec1-Ddc2 to trigger DNA damage checkpoint activation *in vivo*. Studies in yeast have also shown a strong connection between the exposure of ssDNA at DSB and unprotected telomeres and activation of DNA damage checkpoint (Garvik et al., 1995; Lee et al., 1998). At DSBs ssDNA is generated by 5' to 3' resection, leaving long 3'-ended tails (Garvik et al., 1995 (White and Haber, 1990).

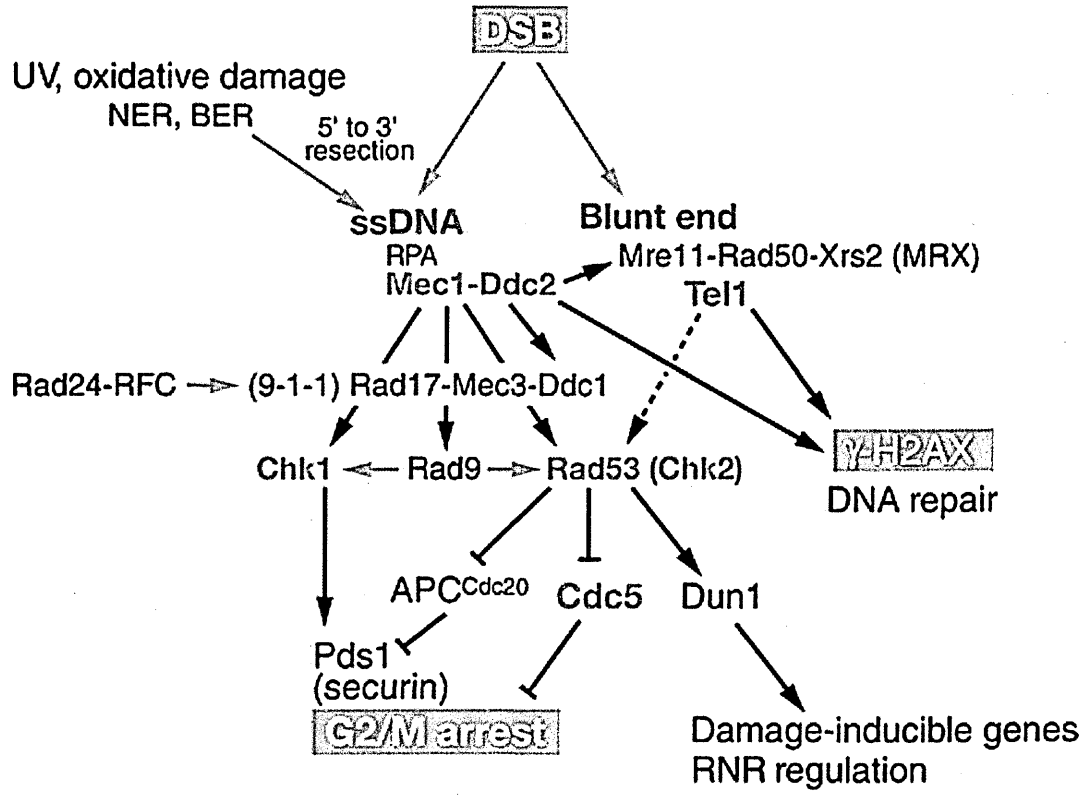


Figure I. Schematic representation of checkpoint pathway in *S. cerevisiae* from Harrison and Haber 2006.

The generation of ssDNA at a DSB requires the activity of a 5' to 3' exonuxclease or a helicase/endonuclease similar to bacterial RecBCD. The budding yeast Mre11-Rad50-Xrs2 (MRX) complex is responsible for some of the activity, as the deletion of any of these proteins results in a two fold reduction in resection rate in cycling cells (Ivanov et al., 1994; Nakada et al., 2004; Tsubouchi and Ogawa, 1998). The MRX complex appears to be regulated by the Sae2 protein that has been recently shown to play a role in the resection of HO-induced DSBs in mitotic cells (Clerici et al., 2005), presumably by regulating an MRX-dependent nuclease activity. Resection of an HO-induced DSB is also partially reduced by deletion of the exonuclease Exo1, and this effect is more pronounced at distance beyond 2.3 kb from the HO-induced DSB (Llorente and Symington, 2004).

Another factor governing the generation of ssDNA at DSB is the phase of the cell cycle. In

budding yeast cells arrested in G1 by the mating type pheromone (wich prevents activation of the B-type cyclins, or "Clbs"), the resection is very slow. A similar block in resection can be achieved by over-expressing the Clb inhibitor Sic1, or by inhibiting the Cdk kinase (Aylon et al., 2004; Ira et al., 2004), thus enforcing the idea that ssDNA is the signal that activates checkpoint response. These conditions also prevent the phosphorylation of Rad53 protein, the central checkpoint signal transducer in budding yeast (Pellicioli et al., 1999). There are over 200 in vitro targets of Cdk1 phosphorylation (Ubersax et al., 2003), including Mre11 and Xrs2, but we have shown that mutation of the Cdk1 phosphorylation sites in Mre11 and Xrs2 did not affect resection and none of the other targets obviously contribute to DSB resection (Ira et al., 2004). Inhibition of Cdk1 in checkpoint-arrested cells is sufficient to stop ongoing resection of a DSB and to turn off Rad53 phosphorylation, suggesting that continuous Cdk1-dependent resection is required for checkpoint maintenance (Ira et al., 2004). Such a requirement for CDK1 activity in triggering and maintaining resection of DSB extremity and consequently checkpoint activation suggests that the mechanism of DNA repair is tightly correlated to the cell cycle phase. In fact, in G1 cells the activity of CDK1 is inhibited by Sic1 inhibitor and resection is low; this correlates with a preference in this phase in repairing a DSB through Non-Homologous-End-Joining (NHEJ). However, in G2, yeast cells preferentially repair DSBs through homologous recombination (HR). Thus, the activity of CDK1 is thought to co-ordinate the choice of repair pathway within cell cycle.

The recruitment of the Mec1-Ddc2 complex to ssDNA generated at a DSB requires the single-strand binding protein RPA (Zou and Elledge, 2003). Similarly, in human cells depletion of the large RPA subunit RPA70 reduces focus formation by ATR and ATRIP after irradiation and reduces phosphorylation of the ATR target Chk1 (Zou and Elledge, 2003). RPA is also required for the interaction between ATR/ATRIP complex and ssDNA *in vitro* (Zou and Elledge, 2003). No other checkpoint proteins are required for Mec1 and Ddc2 to interact with the site of DNA damage (Kondo et al., 2001; Melo et al., 2001; Zou

et al., 2002), demonstrating that the Mec1-Ddc2 complex is an authentic damage "sensor" and that RPA coated ssDNA is the damage signal that triggers checkpoint activation.

The checkpoint activation and cell cycle arrest requires an heteromeric complex (Rad17, Mec3, and Ddc1) that is composed by proteins that show limited homology to PCNA clamp (Venclovas and Thelen, 2000) and is known as "checkpoint clamp". This complex (called also 9-1-1 from the names of human hortologues: Rad9-Hus1-Rad1), is loaded onto DNA by a checkpoint clamp loader, a form of RFC in wich Rad24 instead of Rfc1, forms a complex with the Rfc2-5 subunits (Venclovas and Thelen, 2000). As shown by ChiP analysis the recruitment of Ddc1 and Mec3 requires Rad24 while does not require either Mec1 or Rad53, thus suggesting the 9-1-1 complex is a third independent damaging "sensor" (Kondo et al., 2001; Lisby et al., 2004; Melo et al., 2001).

Transducing and eliciting the checkpoint signal

After detection of DNA lesions by upstream sensors, the signal is amplified and transmitted through a phosphorylation cascade to the effectors. From Mec1 the signal is transmitted to Rad53 and Chk1. Activation of Rad53 requires interaction with phosphorylated Rad9; this interaction seems to promote the oligomerization of the protein that leads to *in trans* autophosphorylation and fully activation of the kinase activity of Rad53. Both *S. cerevisiae* Rad9 and *S. pombe* Crb2 dimerize via C-terminal BRCT motifs, and this dimerization is required for checkpoint functions *in vivo* (Collura et al., 2005; Du et al., 2004; Mochida et al., 2004; Soulier and Lowndes, 1999). *S. pombe* Chk1 is probably activated, like Rad53, through oligomerization and phosphorylation, since addition of a dimerization domain suppresses the UV sensitivity of a Crb2 allele lacking its N- terminus. Following activation of the checkpoint signaling kinases, cell cycle arrest is effected by direct regulation of the cell cycle machinery. Yeast securin, Pds1, is required for normal cell cycle arrest in response to DNA damage. After DNA damage, Pds1 is hyperphosphorylated in a Mec1-, Rad9-, and Chk1-dependent, but Rad53-independent, manner (Cohen-Fix and Koshland, 1997). In the unperturbed cell cycle, Pds1 protein is degraded at the entry into mitosis after being ubiquitinated by the Anaphase Promoting Complex (APC) in complex with its specificity factor Cdc20, but after DNA damage Pds1 is stabilized by phosphorylation that blocks its ubiquitination (Agarwal et al., 2003; Sanchez et al., 1999). Like Chk1, Rad53 regulates Pds1 stability but does so by specifically blocking the interaction between Pds1 and Cdc20 *in vivo*. The molecular mechanism is unknown, but one site on Cdc20 has been identified as a likely substrate of Rad53 phosphorylation (O'Neill et al., 2002). Regulation of Cdc20 protein abundance is also seen in the yeast S-phase checkpoint and spindle-assembly checkpoint, suggesting that Cdc20 is a common regulatory target to prevent anaphase (Clarke et al., 2003; Pan and

Chen, 2004). While Pds1 regulates mitotic entry, Rad53 also inhibits mitotic exit. Rad53 (but not Chk1) is required to maintain CDK activity during the checkpoint arrest and likely does so through inhibition of Cdc5 (Sanchez et al., 1999). This protein inhibits the mitotic exit network, MEN, by inhibiting the complex Bub2/Bfa1 (de Bettignies and Johnston, 2003). Rad53-dependent inhibition of Cdc5 could therefore inhibit progression through mitosis and help maintaining the checkpoint arrest.

DNA damage provokes a significant transcriptional response; the best-characterized aspect of this response is the induction of transcription of ribonucleotide reductase (RNR genes). Some of the regulation of RNR also occurs post-transcriptionally and are largely under the control of the Dun1 kinase which directly phosphorylates Sml1 and targets it for proteolisis (Zhao et al., 2002; Zhao et al., 2001). Maintenance of adequate RNR activity is essential for cell viability, and the lethality of *mec1Δ* and *rad53Δ* can be suppressed by over-expressing an RNR gene, by ablating Sml1(Desany et al., 1998; Zhao et al., 1998).

In addition to regulation of signaling proteins, DNA damage also leads to Mec1- and Tel1-dependent phosphorylation at serine 129 of the histone variant H2AX. Phosphorylated H2AX, termed γ-H2AX, is detected very soon after DNA damage and is found over a large region of chromatin flanking the DSB, approximately 1 Mb in mammalian cells and 50–100 kb in yeast (Shroff et al., 2004). γ-H2AX has been shown to contribute to DNA repair in both fungal and animal cells and is required for full viability of yeast and animal cells in the presence of DNA damaging agents (Celeste et al., 2003; Nakamura et al., 2004; Redon et al., 2003). γ-H2AX also plays a conserved role in the DNA damage checkpoint since yeast or animal cells that cannot generate γ-H2AX display mild checkpoint defects. In both systems it has been proposed that γ-H2AX functions primarily in checkpoint maintenance because in its absence the checkpoint is activated normally but extinguished prematurely (Celeste et al., 2003; Nakamura et al., 2004; Redon et al., 2003). A major role for γ-H2AX is the recruitment of chromatin remodelers, including the Ino80, Rvb1, NuA4, and Swr1 complexes to the DSB (Bird et al., 2002; Celeste et al., 2003; Downs et al., 2004; Morrison

et al., 2004; Paull et al., 2000; van Attikum et al., 2004). However, none of these factors is known to be strongly required for proper checkpoint function (van Attikum et al., 2004). γ-H2AX also recruits cohesin and the Smc5/6 complex to DSBs, and both of these SMC complexes cover a similarly large chromosomal region as γ-H2AX. In yeast the only aspect of DSB repair that appears to be affected by γ-H2AX formation is repair between sisters chromatids. Cells expressing the nonphosphorylatable histone H2A-S129A have normal repair of meiotic DSBs as well as normal HO-induced recombination but exhibit a fourfold reduction in repair of damage caused by IR (Strom et al., 2004; Unal et al., 2004).

A second damage-induced histone phosphorylation, at serine 1 of histone H4, has been seen in budding yeast (Cheung et al., 2005). This phosphorylation appears more slowly than γ-H2AX and depends on casein kinase II (CKII). How CKII is activated by DNA damage is not yet known, nor it is clear whether this modification contributes to DSB repair or to checkpoint-mediated arrest.

Methylated lysine 79 of histone H3 (H3-K79Me) is bound by the Tudor domain of the yeast and human adaptor proteins Rad9 and 53BP1 and contributes to their recruitment to DSBs (Huyen et al., 2004; Wysocki et al., 2005). In budding yeast, ablation of H3-K79Me by deletion of the Dot1 methyltransferase results in defects in several checkpoints, presumably due to the impaired recruitment of Rad9.

In fission yeast, methylation of lysine 20 of histone H4 (H4-K20Me) also contributes to the checkpoint response by recruiting of the Crb2 adaptor protein to damaged DNA (Sanders et al., 2004). Neither H4-K20Me in *S. pombe* nor H3-K79Me in *S. cerevisiae* is stimulated by DNA damage but exists at a basal level in normal chromatin (Sanders et al., 2004; van Leeuwen et al., 2002). This suggests that the recruitment of Rad9-related adaptors by these histone modifications may require local chromatin decondensation for exposure (Sanders et al., 2004). Acetylation of histone H3 lysine 56 (H3-K56Ac) may also promote DNA accessibility to chromatin, though in this case the relevant targets appear to be repair rather than checkpoint proteins (Masumoto et al., 2005). Recent work in mammalian cells has

shown that γ-H2AX directly interacts with the checkpoint adaptor protein MDC1, a BRCT domain-containing protein that may function analogously to Rad9 (Lou et al., 2006; Stucki et al., 2005).

The NuA4 histone acetyl-transferase (HAT) is recruited in a region up to 1.5 Kb from the break through the interaction of its Arp4 subunit with γH2A where it promotes acetylation of H4-K8 (Downs et al., 2004). Mutations in the catalytic subunit of NuA4 (Esa1) or in the target lysine of histone H4 result in hypersensitivity to DNA damaging agents suggesting that acetylation of H4 by NuA4 is an early event in the DSB response that might be important for the recruitment of other repair factors (Bird et al., 2002; Downs et al., 2004). Reinforcing the role of histone acetylation in the DSB response, a part from NuA4, also Gcn5 (HAT) and Rpd3, Sir2, Hst1 histone deacetylases (HDAC) are recruited during DSB repair at the MAT locus to coordinate waves of acetylation and deacetylation of histones H3 and H4 many of them likely involved in repair through homologous recombination (Tamburini and Tyler, 2005).

Nucleosome removal by ATP-dependent chromatin remodeling factors seems to play also an important role in DSB repair. INO80 complex is recruited to DSB through interaction of multiple subunits with γH2A. Its recruitment is delayed with respect to NuA4 and is required for efficient loading of Rad51 and Rad52 repair proteins although it's not clear whether this is mediated also by a defect in accumulating ssDNA at the break (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). Together with INO80 also SWR1 and SWI/SNF complexes have been detected at DSBs in yeast and involved respectively in facilitating repair through NHEJ and HR, supporting the view that DSB response is highly coordinated with the chromatin remodeling machinery (Chai et al., 2005; van Attikum et al., 2007). The recent finding that Ies4 subunit of INO80 is phosphorylated in response to DNA damage in a Mec1/Tel1 dependent manner, and that such modification is important for the checkpoint signaling, establishes a chromatin-

remodeling complex as a component of the damage response pathway (Morrison et al., 2007).

In summary, several different chromatin modifications contribute to checkpoint arrest and are implicated in the recruitment of checkpoint adaptor proteins to DNA. Whether these modifications play an authentic role in checkpoint maintenance or a partially redundant role in checkpoint activation is still unknown.

Although ssDNA is required to activate the checkpoint, it is not sufficient to maintain it in fact after the checkpoint has been activated by a single DSB, Rad53 hyperphosphorylation disappears when ongoing 5' to 3' resection is arrested by Cdk1 inhibition, despite the continuing presence of extensive ssDNA (Ira et al., 2004). Continuous Mec1 activity is also needed for checkpoint maintenance as Mec1 inactivation either by the PIKK inhibitor caffeine or through use of a Mec1-degron releases the checkpoint arrest (Pellicioli et al., 2001; Vaze et al., 2002).

Switching checkpoint signal off

Adaptation

In the presence of an unrepairable DSB, yeast cells undergo a long checkpoint arrest lasting 12–14 h but then re-enter the cell cycle, or "adapt," despite the persistence of unrepaired DNA (Lee et al., 1998; Sandell and Zakian, 1993; Toczyski et al., 1997). Several proteins are required for adaptation, and their mutations prevent Rad53 inactivation and cell-cycle re-entry. Many of these proteins function in chromatin regulation and recombination, such as Yku70 and Yku80, the Swi2/Snf2/Rad54 homolog Tid1, Rad51, the Srs2 helicase, and Sae2. Others have a checkpoint or a mitotic roles, such as the PP2C-family phosphatases Ptc2 and Ptc3, the CKII subunits Ckb1 and Ckb2, and the Polo kinase Cdc5 (Clerici et al., 2006; Lee et al., 1998; Lee et al., 2001; Lee et al., 2003a; Leroy et al., 2003; Pellicioli et al., 2001; Vaze et al., 2002). The adaptation defect in *yku70Δ* cells is apparently the result of increased resection at an unrepairable DSB and is comparable to that seen in cells resecting two DSBs at a normal rate (Lee et al., 1998). Reducing this resection by deletion of Mre11 suppresses the *yku70Δ* adaptation defect, suggesting that the rate or extent of resection contributes to maintenance of the checkpoint signal and therefore to adaptation (Lee et al., 1998). No other adaptation mutant is known to have increased resection, thus, suggesting that a variety of factors govern adaptation. Dephosphorylation of checkpoint proteins clearly contributes to adaptation, and the phosphatases Ptc2 and Ptc3 are responsible for at least one important dephosphorylation event (Leroy et al., 2003). Ptc2 interacts with the FHA1 domain of Rad53 and presumably inactivates Rad53 by dephosphorylation (Leroy et al., 2003). Ptc2 phosphorylation by CKII (which includes the Ckb1 and Ckb2 subunits) promotes its interaction with Rad53 in

vitro, and these interactions likely explain the adaptation and recovery roles of Ptc2, Ptc3, and CKII (Leroy et al., 2003).

In strains suffering an unrepairable DSB, Ddc2-GFP foci are maintained during the entire checkpoint arrest. At the time of adaptation, however, these foci show reduced intensity and in many cases disappear (Melo et al., 2001). In contrast, Ddc1-GFP foci do not dissociate but maintain intensity or brighten during and beyond adaptation (Melo et al., 2001). These results suggest that the regulation of Mec1-Ddc2 rather than the 9-1-1 clamp is likely to govern the timing of adaptation. One possible Mec1 regulator in this process is Sae2. As mentioned above, Sae2 promotes the dissociation of the MRX complex from DNA. *sae2Δ* cells, which frequently fail to adapt, maintain Rad53 phosphorylation in the presence of a single DSB and can do so in the absence of either Mec1 or Tel1 (but not both). Additionally, overexpression of Sae2 can override the checkpoint arrest following UV irradiation and can do so in the presence or absence of Tel1 (Clerici et al., 2006). These results suggest that Sae2 may function to inhibit Mec1-Ddc2, perhaps by removing the complex from DNA.

The essential role of the DNA damage checkpoint is to prevent the segregation of broken or damaged chromosomes. Adaptation promotes the mis-segregation of acentric chromosome fragments in 95% of divisions, and the mis-segregation of even centric chromosome fragments is seen in 42% of divisions (Kaye et al., 2004). This clearly leads to increased genomic instability as has been demonstrated for both chromosome loss and translocations (Galgoczy and Toczyski, 2001). Despite these phenotypes, adaptation is required for full viability of yeast cells in response to persistent DNA damage, suggesting that very slow or delayed repair of DNA damage, even after adaptation, aids cell viability (Galgoczy and Toczyski, 2001).

Because it promotes genomic instability, adaptation has been considered unlikely in metazoans, but recent work in the *Xenopus* egg extract has demonstrated adaptation to the S-phase checkpoint. In response to the replication inhibitor aphidicolin, cell cycle arrest is

mediated by ATR-dependent activation of Chk1 with the assistance of the adaptor protein claspin. ATR also phosphorylates claspin, and this phosphorylation facilitates the interaction between claspin and the Polo kinase Plx. Plx then phosphorylates a neighboring site on claspin, which promotes claspin's dissociation from chromatin and the attenuation of Chk1 signaling that allows adaptation (Yoo et al., 2004). Whether the yeast Polo kinase Cdc5 contributes to adaptation by a similar mechanism is unknown.

Recovery

When DSB repair is successful, cells turn off the checkpoint and re-enter the cell cycle in a process termed checkpoint recovery. Genetic analysis has shown that many adaptation-defective mutants, including *yku70Δ*, *tid1Δ*, and *cdc5-ad*, are not defective in recovery (Vaze et al., 2002). Some adaptation mutants exhibit slow recovery (ckb1Δ, ckb2Δ, and rad51Δ) (Vaze et al., 2002), but only *srs2Δ*, *ptc2Δ ptc3Δ*, and *sae2Δ* have a strong recovery defect (Vaze et al., 2002, Leroy et al., 2003). Biochemical analysis of the Srs2 helicase shows that it can remove Rad51 from ssDNA *in vitro*, and deletion of Rad51 substantially alleviates *srs2Δ* recovery defect (Krejci et al., 2003; Vaze et al., 2002; Veaute et al., 2003). One possibility is that Rad51 remains on DNA in *srs2Δ* mutant cells, even after successful repair, and promotes maintenance of the DNA damage checkpoint signal through an unknown mechanism. Given that DSB repair products are intact and apparently lack ssDNA (Vaze et al., 2002), it is possible that Rad51 is associated with dsDNA.

In contrast, the PP2C-family phosphatases Ptc2 and Ptc3 (and perhaps CKII) work at the level of Rad53 to extinguish the checkpoint signal. The human homolog of Ptc2 and Ptc3, Wip1/PPM1d, has also been implicated in checkpoint recovery. Wip1 expression is induced after DNA damage in a p53-dependent manner, and Wip1 subsequently reverses PIKK-mediated phosphorylation of both p53 and Chk1 (Lu et al., 2005). Depletion of Wip1 leads to prolonged phosphorylation of both p53 and Chk1 after DNA damage and

prolongs the checkpoint arrest by maintaining inhibition of Cdc2 (Lu et al., 2005). Similarly, depletion of the human Polo kinase Plk1 also impairs checkpoint recovery (van Vugt et al., 2004). Plk1 promotes the degradation of the CDK-inhibitory kinase Wee1, and thereby allows Cdc2 activation and mitotic entry after successful DNA repair (van Vugt et al., 2004). The activity of Plk1 is known to be inhibited by DNA damage (Smits et al., 2000), and it will be of great interest to determine whether regulation of Plk1 (and perhaps Cdc5) activity as DNA repair is completed governs the timing of checkpoint recovery.

In *S. pombe*, the PP1-family phosphatase Dis2 controls the timing of checkpoint recovery by dephosphorylating and inactivating Chk1 (den Elzen and O'Connell, 2004). Dis2 phosphatase activity is not obviously regulated by DNA damage (den Elzen et al., 2004). This suggests that basal Dis2 activity promotes checkpoint inactivation only when the acute stage of checkpoint activation (and presumably Rad3 activation) is over. In budding yeast, PP1 governs recovery from a checkpoint monitoring repair of meiotic DSBs (Bailis and Roeder, 2000; Hochwagen et al., 2005). How PP1 promotes adaptation or recovery is unclear, though dephosphorylation of Rad53, Rad9, or Chk1 is an obvious possibility. *Xenopus* PP1 promotes mitotic entry by dephosphorylation of the CDK activator Cdc25 (Margolis et al., 2003), but the budding yeast homolog of Cdc25, Mih1, is unlikely to be relevant to recovery as it is not known to participate in any aspect of the DNA damage checkpoint.

Dephosphorylation of γ-H2AX also influences the duration of the checkpoint. Studies in yeast have identified a novel, evolutionarily conserved PPP4C phosphatase complex, consisting of Pph3, Psy2, and Ybl046w, that dephosphorylates γ-H2AX *in vitro* and in vivo (Gingras et al., 2005; Keogh et al., 2006). Cells lacking any of these subunits have excess γ-H2AX even in the absence of DNA damage and show persistent γ-H2AX foci in irradiated cells. Additionally, the DNA damage checkpoint is significantly prolonged despite normal DSB repair (Keogh et al., 2006). Detailed studies have shown that γ-H2AX is removed from chromatin during homologous repair of a DSB in both wild-type and

pph3Δ cells. Whether this γ-H2AX maintains checkpoint activity while soluble, or whether it is reincorporated into chromatin at other loci has not yet been determined (Keogh et al., 2006). Studies in human cells have identified the PP2A phosphatase complex as the relevant γ-H2AX phosphatase. Unlike in yeast, however, the prolonged checkpoint in mammalian cells with excess γ-H2AX is apparently due to defects in DNA repair (Chowdhury et al., 2005).

An allele of *S. pombe* Cdc20, slp1-362, was identified as a recovery mutant that specifically prevents recovery after UV irradiation but not HU arrest (Matsumoto, 1997), further underscoring the importance of Cdc20 regulation in cell cycle arrest and re-entry.

Rad53

Rad53 protein contains two FHA domains that are required for interaction with phosphopeptides; during checkpoint activation FHA of Rad53 mediate interaction with PIKK-posphorylated Rad9 leading to catalytic activation of Rad53 and extensive Rad53 autophosphorylation (Durocher et al., 2000b; Sun et al., 1998). The two FHA domains of Rad53 are only partially redundant for its activation. In DNA damage checkpoint assays loss of either FHA domain shortens the normal arrest time, and the double FHA1, 2 mutant is as strongly checkpoint-defective as the Rad53-kd (kinase-dead) allele (Pike et al., 2003; (Schwartz et al., 2003). Mutation of just the FHA2 domain, which strongly interacts with Rad9, reduces Rad53 phosphorylation and the Rad53-Rad9 interaction in MMS-treated cells but not in HU-treated cells (Schwartz et al., 2003; Sun et al., 1998). Mutation of FHA1, which binds more strongly to Rad53 itself and to the S-phase regulators Asf1 and Dbf4, slightly sensitizes cells to HU and impairs the S-phase checkpoint (Duncker et al., 2002; Schwartz et al., 2003; Sun et al., 1998).

Rad53 FHA domains are likely interacting with a cluster of 7 SQ/TQ motifs in Rad9's central region, and mutation of the first 6 of these is sufficient to prevent Rad9 phosphorylation, Rad9-Rad53 binding, Rad53 activation, and checkpoint arrest in damaged cells (Schwartz et al., 2002). Why the checkpoint is not governed by a simple interaction between Mec1 and Rad53 is unclear, but presumably the elaborate activation mechanism requiring Rad9 and the 9-1-1 complex allows greater regulatory flexibility and may reduce spurious signaling.

Recent *in vitro* studies have clearly demonstrated the adaptor function of Rad9 in the phosphorylation of Rad53 by Mec1 (Sweeney et al., 2005). Two studies have also mapped a large number of Rad53 phosphorylation sites before and after damage using mass spectrometry techniques (Smolka et al., 2005b, Sweeney et al., 2005). The two studies did

not find many of the same phosphorylated sites after damage. This could be due to different detection techniques or different damage agents (MMS vs 4NQO) (Sweeney et al., 2005; Smolka et al., 2005b). Several predicted CDK target sites on both Rad9 and Rad53 are also phosphorylated *in vivo*, even in the absence of DNA damage (Smolka et al., 2005b, Sweeney et al., 2005). We have previously argued that the rapid inactivation of Rad53 that follows CDK inhibition is the result of blocked resection (Ira et al., 2004), but it is also possible that direct phosphorylation of Rad9 or Rad53 by Cdk1 contributes to normal checkpoint activation.

A different approach to identify phosphorylation sites required for Rad53 activation has been to mutate the clusters of SQ and TQ residues that are found in Rad53 (Lee et al., 2003b). Rad53 contains an N-terminal cluster of TQ sites and a C-terminal cluster of SQ sites. Both the TQ and SQ motifs contribute to Rad53 phosphorylation in vivo, and mutation of both clusters eliminates most phosphorylation of Rad53 by Mec1 in vitro (Lee et al., 2003b). The TQ phosphorylation cluster interacts with the FHA domain of its signaling target Dun1 and is essential for Dun1 phosphorylation. The TQ cluster is also bound by the Rad53 FHA1 domain, presumably to promote Rad53 oligomerization and activation (Bashkirov et al., 2003; Lee et al., 2003b). Both the SQ and TQ phosphorylation clusters also contribute to Rad53 autophosphorylation in response to DNA damage stimuli (Lee et al., 2003b). Thus, PIKK-mediated (and perhaps CDK-mediated) phosphorylation serves several functions in Rad53 activation: activation of Rad53 kinase activity, promotion of oligomerization and trans-autophosphorylation, and creation of an interface for the Rad53-Dun1 interaction (Lee et al., 2003b; Schwartz et al., 2002).

Activated Rad53 also interacts with the nuclear import factors Srp1 and Kap95, and a major damage-induced phosphorylation site is found within Rad53's bipartite NLS (Smolka et al., 2005b). Mutation of this site prevents the threefold increase in Rad53 levels that is observed after MMS treatment. This suggests that Rad53 activation in part requires nuclear import and promotes Rad53 accumulation (Smolka et al., 2005b).

The figure II from Pellicioli and Foiani 2005, summarizes the results of these two studies.

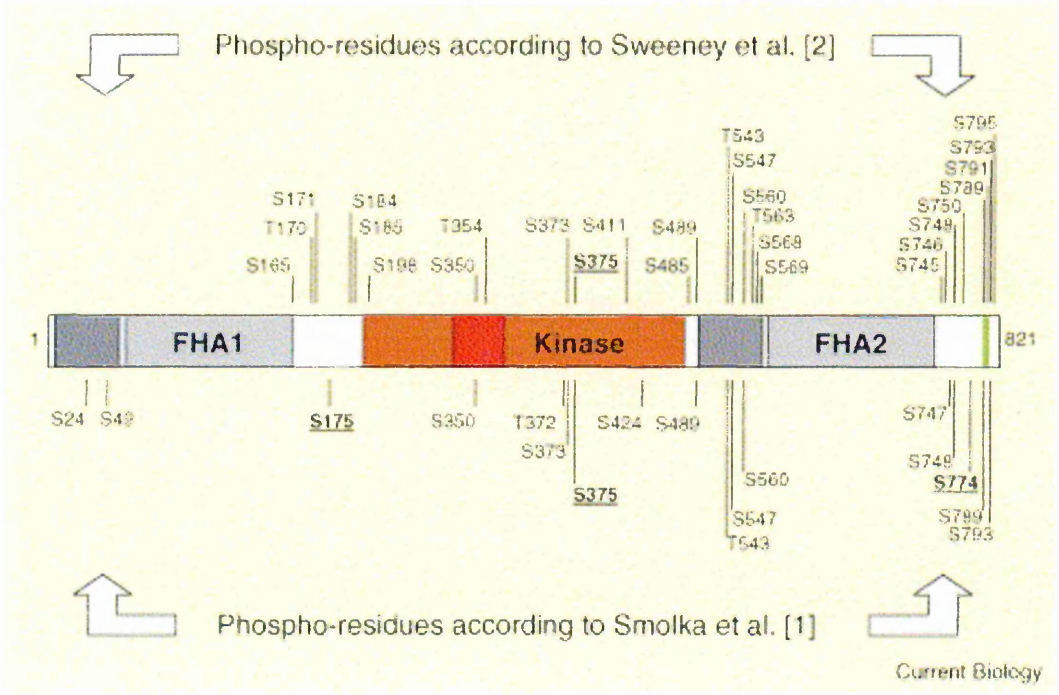


Figure II. Schematic representation of phosphorylation sites along Rad53 sequence.

The T-loop domain

Protein kinases constitute one of the biggest gene families in eukaryotes; despite the conservation of the overall fold of these proteins, differences in the core sequences and flanking regions of kinases can be fine tuned to allow each kinase to respond to a unique set of signals to turn their activity on or off. The spatial and temporal control of phosphorylation of a specific serine, threonine, or tyrosine residues is crucial to cellular growth and development, and this control relies on the proper regulation of the kinases.

Kinase activity in the wrong place or at the wrong time can have disastrous consequences, often leading to cell transformation and cancer.

For many kinases, activation requires phosphorylation of the activation segment. The

kinases that are activated through activation segment phosphorylation have a conserved arginine preceding the conserved catalytic aspartate in the catalytic loop (Johnson et al., 1996) and for this reason are termed RD kinases. It has been proposed that phosphorylation of residues in the T-loop have the role of introducing negative charges that counteract the positive charge of the arginine and the other aminoacids in the catalytic region contributing thus to the correct conformation of the catalytic and of the substrate-interacting domains. This idea is further supported by two observations: kinases that do not possess the arginine before catalytic aspartic, are not phosphorylated in the T-loop, and RD-box containing kinases that are not phosphorylated in the T-loop posses negatively charged aminoacids that could predispose the kinase in an always active state.

The activation segment or loop (from the secondary structure) is defined as the region between and including two conserved tripeptide motifs (DFG...APE). Interestingly the activation loop showed considerable structural diversity as was shown by the comparison of emerging structural data coming from crystallization experiments; this diversity in both conformation and sequence is probably reflecting the requirement for fine tuning of kinase-specific functions. Most kinases contain specific consensus sites within the activation loop that are important for phosphorylation and dephosphorylation, and are critical in determining the conformation of the loop and, consequently, the activity of the kinase. the activation loop is also a site for protein-protein interactions that can be critical in controlling the localization and regulation of a kinase and its binding partners.

Structural evidences have suggested that activation segment is able to undergo large conformational changes when the kinase switches between inactive and active states (reviewed in (Hutchins and Clarke, 2004). For example Johnson and co-workers showed that in the insulin receptor kinase (Irk) the phosphorylation of the activation segment causes the movement of this loop away from the catalytic center and adopt a conformation that allows substrate binding and catalysis that are, in the inactive state, inhibited by the presence of the loop itself.

In the case of CDKs, the fully activation is reached by the interaction with cyclins; this interaction causes a conformational change that causes the moving of the t-loop from the catalytic site and this structure is further stabilized by phosphorylation inside activation segment.

DNA damage and genomic instability

The basis of genomic instability is unfaithful transmission of genetic information from a cell to his daughters. This arises from failure of cellular functions that ensure the accurancy of DNA replication, repair, or mitotic chromosome distribution. Specific functional defects can be associated with a characteristic pattern of genomic instability. For example, inactivation of functions that increase the fidelity of DNA replication or eliminate mutagenic DNA lesions enhances the rate of subtle DNA sequence alterations. This is illustrated by the phenotypes of post-replicative mismatch repair (MMR) or nucleotide excision repair (NER) defects. Malfunctions on MMR pathways have been associated to hereditary nonpolyposis colon cancer. These cells are characterized by a mutator phenotype, an instability of microsatellite sequences and increased levels of somatic recombination. Moreover, mismatch repair deficient cells display also varying levels of tolerance to DNA damaging agents and are thought to be involved in the cell killing mediated by these agents (Jiricny, 1998).

The consequences of a defect in one of the NER proteins are apparent from three rare recessive photosensitive syndromes: Xeroderma pigmentosum (XP), Cockaine syndrome (CS) and the photosensitive form of the brittle hair disorder trichothiodystrophy (TTD). Cell-fusion experiments have led to the identification of seven complementation groups within the NER-deficient class of XP patients (designated XP-A to XP-G), two in CS (CS-A and CS-B), three in the category of patients with combined XP and CS (XP-B, XP-D and XP-G) and also three in TTD (XP-B, XP-D and TTD-A). Each of these groups reflects a defect in a distinct gene. It is remarkable that different mutations in the *XPB*, *XPD* and *XPG* genes are associated with a specific clinical outcome: either XP, or XP/CS or TTD (for XPB and XPD). Importantly, sun-sensitive skin is associated with skin cancer predisposition in the case of XP, but not in CS and TTD.

Inactivation of specific checkpoint functions leads to cancer predisposition syndromes.

For example inactivation of ATM gene causes Ataxia Telangiectasia syndrome or AT-related syndromes. All these syndromes are autosomic recessive syndromes associated with immunodeficiency, iper-sensitivity to IR and cancer predisposition.

Mutations on *NBS1* gene cause Nijemen breakage syndrome, characterized by ionizing-irradiation sensitivity, a failure to arrest the cell cycle at G1/S in response to DNA damage, chromosomal instability, and cancer predisposition.

The Werner syndrome (WS) is characterized by premature aging, genomic instability, chromosomal breakages, deletions and translocations and is caused by mutations that affect WRN gene. The Wrn protein has an ATP-dependent, DNA dependent helicase activity and an exonucleolitic activity. The C-terminal portion interacts with Ku86/70 complex and this interaction stimulates the helicase activity. Different studies have shown that the complex can bind ssDNA as well as gaps on the double helix, suggesting its implication with Wrn in recombination and repair processes.

Mutations of the BS genes are associated to BS syndrome resulting in cancer predisposition and genomic instability, immunodeficiency, and infertility. Those characteristics are associated to the inability of cells carrying mutations in this gene, in solving replication intermediates. This defect is associated with high mitotic recombination rates.

The BS gene codes for a DNA helicase belonging to the same family as Wrn. A possibile role for this protein is to prevent the processing of replication intermediates or the replication fork collapse due to a pausing in replication fork progression in physiological slow replicating zones or even as a consequence of a DNA damage.

MATERIALS AND METHODS

Yeast transformation

The procedure used is described by (Gietz et al., 1995), with some modifications.

Exponentially growing cells are treated for 30-60 min with Lithium Acetate (LiAc) 0.1 M in TE 1X. A 100 µl aliquot corresponding to 10^8 cells is incubated with 2-5 µg linear DNA cassettes or 1 µg of plasmid DNA and 20 µg of ssDNA carrier for 30-60 min. 5 volumes of 40% PEG-4000 (Polyethylene Glycol) are added and cells are incubated 30-60 minutes more at RT. Next cells are heat shocked for 10-12 minutes at 42°C and plated in selective plates.

Strains (genotypes) used:

cy5926 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ rad53T354AT358A::KanMX4*

cy5841 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 KanMX6::rad53K227A*

cy5923 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354AT358A::KanMX4*

cy5924 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354DT358D::KanMX4*

cy5943 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354AT358A::KanMX4 <prad53SS, CEN, LEU2>*

cy5945 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ rad53T354AT358A::KanMX4 <prad53SS, CEN, LEU2>*

cy5984 *Matα, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112,lys5,ura3-52,ade3::GAL::HORAD9-13MYC::TRP1,rad53::rad53T354AT358A::KAN*

cy5986 *Matalpha, hmldelta::ADE1, hmrdelta::ADE1 ade1-100, trp1delta::hisG, leu2-3, leu2-112,lys5,ura3-52,ade3::GAL::HO,DDC2-HA3::URA3 rad53::rad53T354AT358A::KAN*

cy6166 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, rad53T354DT358D::KANMX4*

cy7094 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS rad53K227A::KANMX4*

cy7096 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS rad53AA::KANMX4*

cy7171 *Matalpha, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS, rad53T354DT358D::KanMX4*

cy7599 *MATa, ade2-1, trp1-1, leu2-3112, his3-1115, ura3, can1-100 GAL PSI1+ rad53T354DT358D::KANMX4, rad52::TRP1*

cy7602 *MATa, ade2-1, trp1-1, leu2-3112, his3-1115, ura3, can1-100 GAL PSI1+rad53T354DT358D::KANMX4, sml1Δ::HISMX6, rad52::TRP1*

cy7657 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ rad53T354AT358A::KanMX4 DUN1-HA-TRP*

cy7697 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ rad53K227A::KanMX4 DUN1-HA-TRP*

cy7702 *MATa, ade2-1, trp1-1, leu2-3112, his3-1115, ura3, can1-100 GAL PSI1+rad53T354DT358D::KanMX4, DUN1-HA-TRPE2::URA3, MRE11::TRP*

cy7726 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS rad53T354DT358D::KanMX4Matalpha, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+*

cy7730 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS rad53K227A::KANMX4Matalpha, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+*

cy7732 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS rad53AA::KANMX4Matalpha, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+*

cy5976 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ DUN1-3HA::TRP1*

cy5976 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ DUN1-3HA::TRP1 GALRAD53@LEU2*

cy5977 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ DUN1-3HA::TRP1 GALrad53D339A@LEU2*

cy5978 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ DUN1-3HA::TRP1 GALrad53T383AT387A@LEU2*

cy5979 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ DUN1-3HA::TRP1 GALrad53T383DT387D@LEU2*

cy2034 *Mat a, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+,rad53K227A KAN*

cy5833 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+*

cy3146 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6*

cy7075 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ SML1-3HA::HIS*

cy5924 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354DT358D::KanMX4*

cy5943 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354AT358A::KanMX4 <prad53SS, CEN, LEU2>*

cy5944 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354DT358D::KanMX4 <prad53SS, CEN, LEU2>*

cy5942 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+ rad53T354AT358A::KanMX4 <pRAD53, CEN, LEU2>*

cy5941 *Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, sml1Δ::HISMX6 rad53T354DT358D::KanMX4 <pRAD53, CEN, LEU2>*

Total protein extract

The yeast protein extraction was performed with Tri-chloroacetic acid (TCA) method as described by (Reid and Schatz, 1982). The method is very efficient to avoid proteolytic phenomena during cell lysis.

Starting sample 0.5-2 x10^8 cells.

Cells are blocked with 2mL of TCA 20%, vortex and transfer to 2 ml tube. The pellet is re-suspended in 200 µL of TCA 20% and an equal volume of acid-washed glass beads (425-600 µm, Sigma-Aldrich) is added. Cells are broken by continuous vortexing for 2-4 minutes. 200 µL of TCA 5% is added to have a final concentration of 10% of TCA. The lysate is transferred to a new 1,5 ml tube and centrifuged for 10 minutes at 3000rpm, RT. The pellet is re-suspend in 100 µL Laemly Buffer 1x. The pH is neutralized with 50 µl of Tris Base 1 M. Then the protein extract is boiled for 3 minutes and centrifuged for 10 minutes at 3000rpm, RT. The supernatant is collected and analyzed through SDS-PAGE.

EL7 monoclonal antibodies were used in western blot against Rad53p.

FACS (Fluorescence activated cell sorter) analysis

0.5-2 x10^8 cells are blocked with 70% ethanol in Tris 250mM pH7.6. Cells were treated with 2 mg/ml RNAse A in Tris 50mM pH7.6 for 1hour at 37°C. Next cells are stained with Propidium Iodide 50 µg/ml in 180 mM Tris-HCl (pH 7.5); 190 mM NaCl; 70 mM MgCl2. A 1:10 dilution in Tris 50mM (pH7.6) is analyzed in Becton Dickinson FACS-calibur for FL2H fluorescence.

Genomic DNA extraction with the Qiagen genomic Kit

Materials and Solutions

Solutions Y1, G2, QBT, QC and QF are supplied with the Qiagen Kit:

-*Buffer Y1* (Yeast lysis buffer) 1 M sorbitol; 100 mM EDTA; 14mM β-mercaptoethanol (to be supplemented with 500 µL/sample of zymolyase solution)

-Zymolyase solution, 10 mg/ml (1000 U/ml)

-*Buffer G2* (Digestion buffer) 800 mM guanidine HCl; 30 mM Tris-Cl pH 8.0; 30 mM EDTA pH 8.0; 5% Tween-20; 0.5% Triton X-100

-*Buffer QBT* (Equilibration Buffer) 750mM NaCl, 50 mM MOPS pH 7.0; 15% Isopropanol; 0.15% Triton X-100

-*Buffer QC* (Wash Buffer) 1.0 M NaCl; 50 mM MOPS pH 7.0; 15% Isopropanol

-*Buffer QF* (Elution Buffer) 1.25 M NaCl; 50 mM Tris-Cl pH 8.5; 15% Isopropanol

-RNase (DNA-se free), 10mg/ml

-Proteinase K, 20mg/ml

- Isopropanol

-70% Ethanol

-10mM Tris-HCl pH 8.0

-*Corex glass tubes*

Procedure

1) Start from 1 X 10^9 cells (200 ml of a culture 1 X 10^7 cells/ml

2) Collect samples by centrifugation at 6000-8000 rpm (JA-14 Beckman tubes), 5-10 min, at 4°C, washed once with 20ml cold water.

3) Transfer cells in 50 ml Falcon tube, re-suspend in 5 ml of buffer Y1 plus 500 µL of Zymolyase solution and incubate for 20-30 minutes at 30°C (spheroplasts preparation)

4) Collect spheroplasts by centrifugation at 4000 rpm (in Falcon tubes) for 10 min at 4°C

5) Empty supernatant and resuspend well sferoplasts in 4 ml of buffer G2 (cell breaking)

6) Add 100 µL of RNAse A 10mg/ml and incubate for 20 minutes at 37°C

7) Add 150 µL of Proteinase K 20mg/ml and incubate for 2 hours at 50°C (the lysate should become clear upon Proteinase K digestion)

8) Centrifugate the lysate for 10 minutes at 4000 rpm (in falcon tubes) at 4°C

9) Dilute the supernatant in an equal volume (4 ml) of equilibration buffer QBT

10) Load on the Qiagen tip 100G anion exchange column, pre-equilibrated with 4 ml of buffer QBT.

11) Wash twice with 7.5 ml of buffer QC.

12) Elute with 5 ml buffer QF in corex glass tubes.

13) Precipitate with 3.5 ml of isopropanol and centrifugate at 8500 rpm for 10 min in a Beckman JS 13.1 swing out rotor.

14) Wash the pellet with 1 ml of ethanol 70%.

15) Resuspend in 250 µL of Tris 10mM pH 8.0 and store at 4°C

Quantification of DNA preps

1-2 μl of DNA preps are quantified using a DNA fluorimeter or using standard gel elechtophoresis. An aliquot of sample, corresponding to 8-10μg of total DNA, is digested with the appropriated restriction enzyme and subjected to neutral-neutral 2D gel electrophoresis.

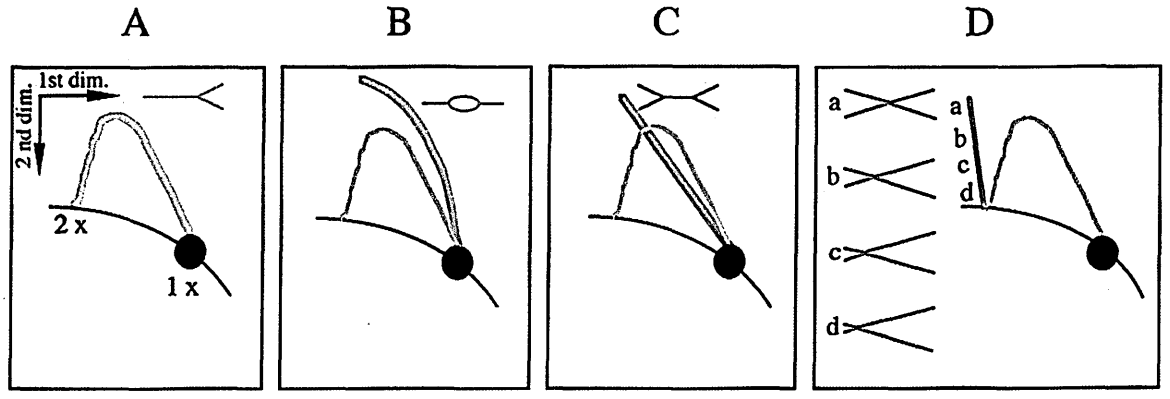
Analyzing replication intermediates by two dimensional agarose gel electrophoresis

When a DNA fragment is being replicated, it assumes different structures that differ from each other by their mass and shape. In particular, when an origin of replication is fired bi-directionally inside the fragment, bubbles shaped structures with increasing mass are formed as the fork proceeds towards the ends. Instead, when a fragment is replicated passively, meaning that a replication fork enters from one extremity, a population of Y-shaped structures with different mass and shape will form.

Neural-neutral two-dimensional agarose gel electrophoresis (2D-gel) technique allows separation and identification of branched DNA molecules according to their mass and shape complexity (Bell and Byers, 1983). This technique further developed by (Brewer and Fangman, 1987) has been used to map origins of DNA replication in yeast chromosomes and to study replication and recombination related DNA structures in many organisms.

The principle of the method exploits the differences in electrophoretic mobility between DNA fragments with the same mass but different shape complexity. Restriction fragments are separated through a first dimension gel, in conditions that minimize the contribution of shape to the mobility (low agarose concentration low voltage no ethidium bromide), virtually by their mass. Subsequently, each lane is cut out and separated through second dimension gel, where DNA runs orthogonally with respect to the first dimension, in conditions that maximize the effect of shape complexity the electrophoretic mobility (high agarose concentration, high voltage and in the presence of ethidium bromide). The features of the 2D-gel pattern are deduced from the expected electrophoretic migration properties of branched molecules and have been in some cases confirmed by electron microscopy

(Kuzminov et al., 1997). In model 1 are represented the migration pattern of classical replication and recombination related branched DNA molecules.



Model 1: Schematic diagrams of 2D gel patterns obtained by analysing a restriction fragment (Lucas and Hyrien, 2000)

Two-dimensional agarose gel electrophoresis

First dimension gel, 0.35% agarose (US Biological-LOW EEO) in TBE1X, without Ethidium Bromide is poured at 4°C.

Samples are loaded in the first dimension alternated with empty lanes to avoid cross-contamination after excision of singular lanes and second dimension.

The first dimension is run at room temperature at 1V/cm for ~24 hours (The conditions are slightly modified depending on the size of the restriction fragment analyzed)

The gel is stained with 0.3 μg/ml ethidium bromide for 30 min.

Slides of appropriate dimension, containing the linear and the replicated size of the fragment of interest are cut from each lane of the first dimension gel.

The slides are placed in the second dimension gel (0.9% agarose with 0.3 μg/ml ethidium bromide) rotated at 90° with respect to the direction of the first dimension.

The second dimension is run for 8-10 hours at 5 Volts/cm at 4°C

Cultures

All the experiments were done in YPD medium containing 10g yeast extract, 20g peptone, glucose 2% final concentration, H_2O to 1 liter; the pH was adjusted to 5.4 with HCL. *E. Coli* strains (DH5α) used for the production of mutants and cloning were growth in LD medium containing 10g Bactotryptone, 5g yeast extract, 5g NaCl, H_2O to 1 liter and the pH was adjusted to 7.25.

Production of integrated versions of Rad53 alleles

The MscI-HpaI fragment of the derivatives pCH10 plasmids generated by mutagenesis (pSF10, pSF11), were sub-cloned into pCH8 or pCH12 plasmid vectors (previously described in (Pellicioli et al., 1999) to obtain the battery of integrative plasmids with the alleles of *RAD53* gene under the control of genomic promoter or Galactose-inducible promoter, respectively (pSF22, pSF23, pSF24, pSF25, see table below for a detailed list). To obtain the yeast strains cy5926, cy5923, cy5924, cy5841, cy5984, cy5986, cy6166, cy5976, cy5977 cy5978, cy5979, the plasmids pSF22, pSF23, pCH8, pSF24, pSF25 and pCH12 were digested with EcoRI or BstXI, respectively.

NAME	GENOTYPE	strain name
pSF1	rad53-T354A-9myc, CEN, LEU2	CB1036
pSF2	rad53-T358A-9myc, CEN, LEU2	CB1037
pSF3	rad53-T354D-9myc, CEN, LEU2	CB1038
pSF4	rad53-T358D-9myc, CEN, LEU2	CB1039
pSF10	rad53-T354AT358A-9myc, CEN, LEU2	CB1045
pSF11	rad53-T354DT358D-9myc, CEN, LEU2	CB1046
pSF12	rad53-T354A, CEN, LEU2	CB1047
pSF13	rad53-T358A, CEN, LEU2	CB1048
pSF14	rad53-T354D, CEN, LEU2	CB1049
pSF15	rad53-T358D, CEN, LEU2	CB1050
pSF16	rad53-T354AT358A, CEN, LEU2	CB1053
pSF17	rad53-T354DT358D, CEN, LEU2	CB1054
pSF22	rad53-T354AT358A, KAN, integrative	CB1064
pSF23	rad53-T354DT358D, KAN, integrative	CB1065
pSF24	GAL1rad53-T354DT358D, LEU, integrative	CB1066
pSF25	GAL1rad53-T354AT358A, LEU, integrative	CB1067
pSF20	rad53-T354AT358A-9myc, CEN, LEU2	CB1068
pSF21	rad53-T354DT358D-9myc, CEN, LEU2	CB1069

Table2. List of plasmids used in this thesis

Production and characterization of monoclonal antibodies against Rad53

Monoclonal antibodies targeting phosphorylated RAD53 were produced as previously described (Harlow and Lane, Antibodies, a laboratory manual, CSH Press), with minor modifications.

Briefly, two 129 mice were boosted intraperitoneally for 7 times with 100 micrograms of phosphorylated RAD53 per injection. Bleedings were taken after the third and the sixth immunization to asses the level of the immune response. The mouse that better responded to immunization was boosted intraperitoneally with 100 micrograms of phosphorylated RAD53 in PBS. After four days, the mouse was sacrificed, the spleen was surgically removed and the splenocytes were fused with SP2/OAg14 myeloma cells. After two weeks, supernatant from wells with growing colonies were tested by ELISA on the

immunogen. Positive wells were subcloned by limiting dilution, and clones were tested by ELISA and Western blot.

Western blot

Proteins are separated by their molecular weight in denaturing condition. The gel is composed of Acrilamide 10% and bis-acrilamide 0.13%. Electrophoresis running is performed in a SDS-PAGE running buffer. The procedure is described by Laemmli (1970). Proteins with a known molecular weight serve as a marker. Proteins are transferred on a nitrocellulose filter (Schleicher and Schuell) by electrophoresis (0.2 Ampere overnight in a transfer buffer (glicin 1%, tris base 0.02M, methanol 20%).

After the transferring filter is washed one time with TBS1X and colored with ponceaus (ponceaus 1gr, AcH 2ml, H2O 200ml) that allow the visualization of all the protein extract. Then the filter is discolored in TBS and incubates 1h in a pre-hybridization solution. This solution is made of milk 4% dissolve in TBS and Tween20 0.2%.

After the pre-hybridization step, primary antibodies are added and 2h of incubation is done at RT.

Afterwards the filter is washed 3 times for 10' with TBS and Tween20 0.2%. At this time the filter is incubated 1h at RT with the secondary antibody. If the primary antibody is a monoclonal antibody an anti-IgG mouse are used, instead if is a polyclonal antibody anti-IgG rabbit are used. These secondary antibodies (Amersham) are conjugated with peroxidase.

After the hybridization with the secondary antibody the filter are washed 3 times for 10' with TBS and Tween20 0.2%.

Finally the filter is washed for 5' with the ECL kit and exposed with a photographic slide in the dark room.

DNA isolation in agarose plugs and PFGE

Pulsed field gel electrophoresis (PFGE) techniques have made possible the resolution of DNA molecules up to several million base pairs in length (Lai, E. et al., 1989; Birren, B.W. et al., 1989). Manipulation of naked DNA of this size in liquid creates double-stranded breaks due to mechanical shear forces. To avoid this fragmentation, DNA can be embedded in an agarose matrix. Intact cells are immobilized in agarose, and then treated to disrupt their cell walls and remove cellular protein. Subsequently, the DNA-containing agarose plug is manipulated in much the same way as DNA in solution and can be subjected to a electrophoresis runs to separate all the individual chromosomes.

Materials and Solutions

- 1% Low Melting Point agarose (LMP) in 0.125M EDTA pH8.0 store at RT.
- 10mg/ml Zymolyase stock (1000U/ml)
- 20% Sarkosyl stock store at RT.
- 10mg/ml Proteinase K stock.
- SCE Solution: 1M sorbitol, 0.1M Sodium citrate, 0.06M EDTA pH8.0.
- Solution I: SCE, 0.2% β-mercapto ethanol, 1mg/ml Zymolyase (100U/ml).
- Solution II: 0.5M EDTA pH8.0, 1% Sarkosyl, 1 mg/ml Proteinase K.
- 1X TE pH8.0.
- Plugs cast from Bio-Rad

Procedure

1. Melt 1% LMP agar and store in a bath at 50°C.
2. Re-suspend the cells in Solution I (50μl for each plug)

3. Add an equal volume of 50°C molten LMP agarose and mix with a pipette. This step needs to be done quickly by putting the eppendorf in a water bath at 50°C; in this way the agarose does not solidify.

4. Cover the bottom of the plug cast with tape.

5. Fill plug cast with cell/agarose mix (approximately 90µl per plug).

6. Put the cast at 4°C for 20-30 min to allow the solidification of the plugs.

7. Eject plugs in a 50ml falcon tube and cover them with Solution I; generally, calculate around 0.5ml for plugs.

8. Leave at 37°C for 1h.

9. Gently remove Solution I and wash the plugs with an abundant volume of 0.5M EDTA pH8.0.

10. Re-suspend the plugs within Solution II (0.5 ml/plug).

11. Leave overnight at 37°C.

12. Discard the Solution II and wash 3 times with an abundant volume of 1xTE pH8.0: you can fill the falcon tube with 1xTE pH8.0 and wash the plugs directly in there. It is important to wash very well to eliminate the detergent.

13. Transfer the plugs in a new 50ml falcon tube and wash for 2h with 1xTE pH8 at RT on a rotating wheel.

14. Transfer the plugs that you do not analyze immediately in 15ml falcon tubes and cover them with 1xTE pH8 at RT. Blocks can be stored indefinitely at 4°C.

15. Transfer the plugs that you wish to analyze in a new falcon tube and equilibrate them for 1h in the running buffer of the gel (0.5X TBE) in the horizontal wheel.

Run condition:

Gel 0.9% in 0.5X TBE

Running buffer 2.5L 9°C 0.5X TBE

Run: 200V I pulse: E/W 90 for 15h

II pulse: N/S 125 for 9h

Ethidium bromide 1:20000 in agitation

For other running conditions see:

A.Lengronne et al., NAR 2001

in-situ kinase assay

Yeast proteins are separated by canonical SDS-page electrophoresis.

Gels have been blotted onto PVDF (Immobilon-P, Millipore) in Towbin 1X+MetOH 20% Bf. (200mA/over night RT; or 1A/1huor 4°C.).

DENATURING STEP: submerge membrane in Denaturing solution (freshly prepared) for 1h./RT. Glass-made oven baths are reccomended.

Discard the denaturing solution and wash the filters with TBS 1X for 2X10min./RT

RENATURING STEP: submerge membrane in renaturing solution (freshly prepared) for 12-18h./4°C. with weak agitation.

Discard the renaturing solution and wash the filters with Tris-HCl 30mM for 30-60min./RT

KINASE RX: suberge menbrane in the kinase Bf. without hot ATP for 15min./RT. The

kinase reaction could be performed in the canonical glass tube for hybridization or in the glass oven baths as well.

Discard the kinase bf. without hot ATP and submerge membrane in the kinase bf. plus hot g-32ATP 10mCi/ml for 1h./RT

WASHING STEPS:

2X10min. Tris-HCl 30mM pH=7.5

1X10min. Tris-HCl 30mM pH=7.5 +NP-40 Nonidet 0.1% (freshly prepared)

1X10min. Tris-HCl 30mM pH=7.5

1X10min. KOH 1M

rinse with water

1X10min. TCA 10%

rinse with water

dry about 15min. onto a 3MM paper and expose (try 2h for the first exposition)

Solutions and reagents:

Denaturing solution (50ml):

7M Guanidinium-chloride (MW 95.53) 33.4gr.

50mM DTT (MW 154.24) 0.385 gr.

2mM EDTA (stock 500mM) 200ml

50mM Tris-HCl pH=**8** (stock 1.5M pH=**8.8**) 1.67ml

Renaturing solution (500ml):

2mM DTT (MW 154.24) 0.154 gr.

2mM EDTA (stock 500mM) 2ml

10mM Tris-HCl pH=**7.5** (stock 1.5M pH=**8.8**) 3.34 ml

140mM NaCl (MW 58.44) 4.091 gr.

1% BSA (add slowly) 5gr.

0.04% Tween 20 (stock 100%) 200ml

Kinase-reaction solution (100ml):

1mM DTT (MW 154.24) 0.015 gr

0.1mM EGTA (stock 500mM) 20ml

20mM $MgCl_2$ (stock 1M) 2ml

20mM $MnCl_2$ (stock 1M) 2ml

40mM Hepes/NaOH pH=8.0 (stock 500mM) 8ml

100mM Sodium OrthoVanadate (stock 1M freshly prepared) 20ml

Sodium OrthoVanadate:

dissolve 92mg of Na_3VO_4 (MW 183.91, 99%) in 1ml 1M Hepes/NaOH pH=7.5. In eppendorf 2ml tube, in a mixer 15min. at 42°C

Transfer Buffer (5l.):

Glicine 55,8 gr.

Tris Base 12,1 gr.

MetOH 1l.

H20 to 5 l.

Production of point mutation alleles of Rad53

In order to produce new Rad53 alleles we took advantage of the site-directed mutagenesis strategy. This technique is based on a PCR reaction with two oligonucleotides carrying the mutation on a plasmid template. The plasmid carries the Rad53 wild type gene under its own promoter as well as the ampicilline gene for the selection in E.coli. The scheme of the sequence variations to obtain conversion from Threonine (T) to Alanine (A) or Aspartate (D) is reported below.

A ◁——	T	——▷ D	MUTANTS
GCT	ACT	GAT	T354A
GCC	ACC	GAC	T358A
GCA	ACA	-	T354D
GCG	ACG	-	T358D

For the substitution from Threonine to Alanine it is necessary to introduce only one base substitution while to obtain mutation to Aspartate it is necessary to introduce two base changes. The PCR reactions conditions have been adjusted to fit these requirements. Primers named "s" are used to sequence the gene, and primers named "R" represent the opposite direction. The mutations introduced are labeled in red.

NAME	POSITION respect to ATG	SEQUENCE
RAD53M1	-499	5' GTGGTATCG GCAACTATAGGACG
RAD53s1	-230	5' CCGTGGGTAGACTTGGAAATG
RAD53s2	-88 R	5' GTCTTCCAATAATCTCTACCGTCC
RAD53s3	100	5' TGCAGGGTCATTTGTACCACG
RAD53s6	225 R	5' GTCACAGGCTGGGTTTCTACC
RAD53s5	320	5' ATGGGACCTGGTTAAATGGGC
RAD53s4	564 R	5' GGCCACCATTGATGATGCAGTAG
RAD53M3	819	5' GGAGTTCGTTTCTGGTGGTGAC
RAD53s7	1003R	5' CCAATACAGGATCGTCTTGTT
RAD53s8	1045	5' GGGTCTTTTATGAAAACCTTCTGTGGC
T354AF	1045	5' GGGTCTTTTATGAAAGCCTTCTGTGGC
T354AR	1071 R	5' GCCACAGAAGGCTTTCATAAAAGACCC
T354DF	1045	5' GGGTCTTTTATGAAAGACTTCTGTGGC
T354DR	1071 R	5' GCCACAGAAGTCTTTCATAAAAGACCC
T358AF	1061	5' CCTTCTGTGGCGCTTTGGCATATG
T358AR	1084 R	5' CATATGCCAAAGCGCCACAGAAGG
T358DF	1061	5' CCTTCTGTGGCGATTTGGCATATG
T358DR	1084 R	5' CATATGCCAAATCGCCACAGAAGG
RAD53s9	1101	5' CAGAGGTAAAGATACATCCG
RAD53s10	1213R	5' GGCCCGTTAGGATAACATAC
RAD53s11	1299	5' CCGGATATCTGAAGAAGCAAG
RAD53s12	1555	5' GAACAGGATCAGGAAGACCAAG
RAD53s13	1883	5' GAGGCATGCTGTAGGCAAAAG
RAD53s14	2131R	5' GTAACATACCTAATCCCTCG
RAD53s15	2191	5' CCCAGATGATGGCAGCTCAAC
RAD53s16	2306	5' CGACTTGGTAGAGTCACCG

Table3. List of primers used in site-directed mutagenesis.

PCR reaction

The PCR conditions are optimized to facilitate the annealing of mutated primers to wild type template; we used an high fidelity polymerase (Pfu) to ensure that the only mutation introduced was the one presents in the primers. Due to the size of the plasmid and polimerization rate of Pfu we set the elongation time to 25 minutes. 4µl of pC10 were added to 8µl of each primer (2µg/ml), 20µl Pfu buffer 10X, 20µl dNTPs (2ng/µl), 8µl Pfu

and H2O to 200µl. the reaction was then split into four tubes to minimize the amplification of mutations due to amplification. The PCR reaction program is reported below.

Program	
1	5' 95°C
2	30" 95°C
3	1'30" 55°C
4	25' 68°C
5	16 times to 2
6	5' 68°C
7	24h 4°C

Digestion and amplification.

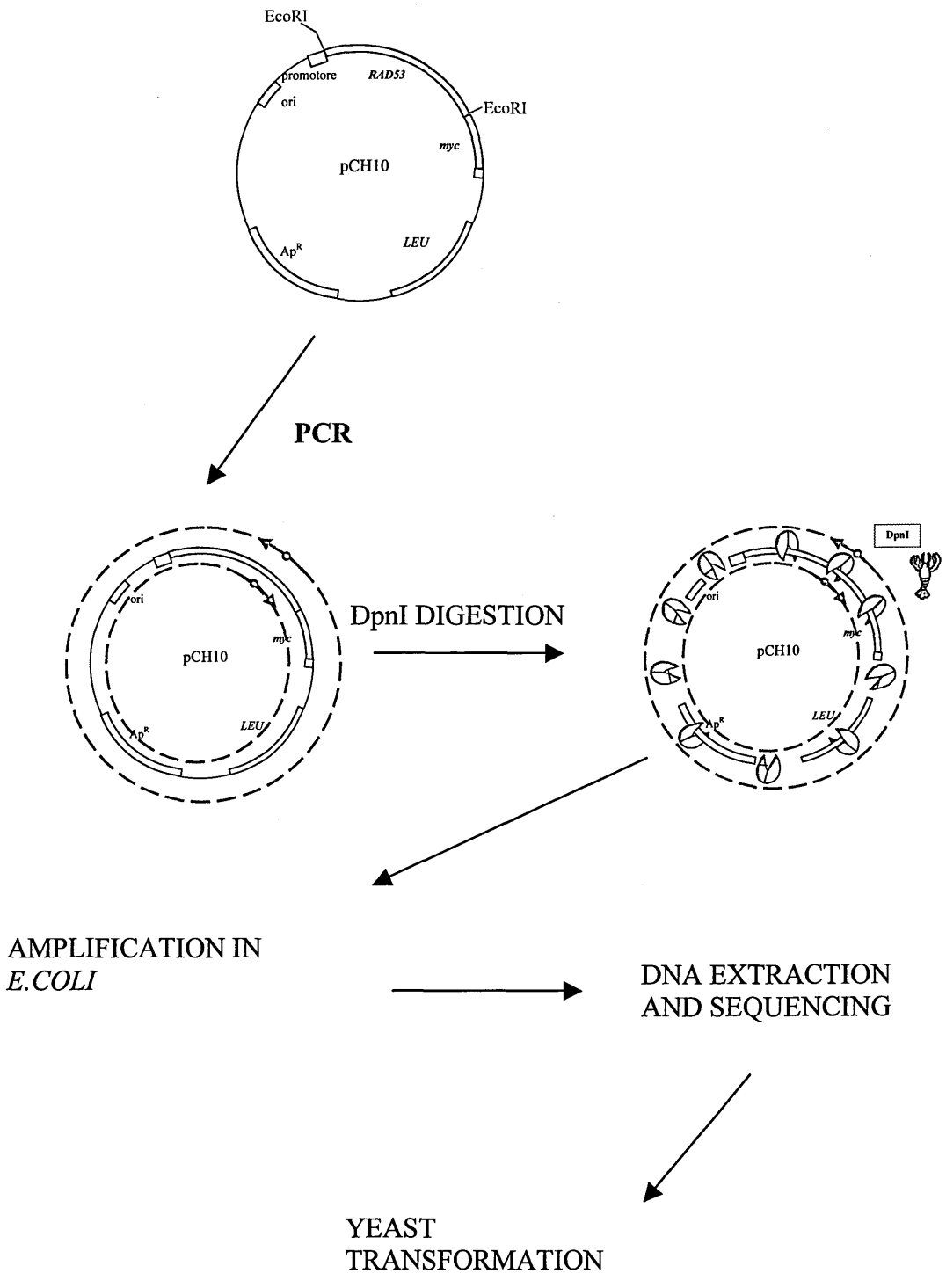
In order to eliminate the wild type template presents in PCR reactions we digested the amplified DNA with DpnI that is sensitive to methylation states of the DNA. In particular this enzyme digest methylated sequences. The template DNA was extracted from *E.coli* and results methylated; the amplified DNA is not methylated. Digestion reaction is performed with 1.5µl (10000U/µl) of DpnI added to 100µl of PCR reaction and incubated 1,5 hours at 37°C. 2-5µl of this digestion were then used to transform *E.coli* DH5α.

Sequencing

DNA was extracted from different clones with Quiagen protocol and analyzed by sequencing with primers reported above to exclude additional mutation in the gene.

Below it is reported a scheme of the entire procedure. This kind of mutagenesis has been previously described in (Hemsley et al., 1989).

The scheme (scheme1) below represents a summary of the technique.


EcoRI
promotore
RAD53
ori
EcoRI
myc
pCH10
Ap^R
LEU
PCR
ori
myc
pCH10
Ap^R
LEU
DpnI DIGESTION
DpnI
ori
myc
pCH10
LEU
Ap^R
AMPLIFICATION IN
E.COLI
DNA EXTRACTION
AND SEQUENCING
YEAST
TRANSFORMATION

RESULTS

Production of monoclonal antibodies directed against Rad53 protein

In this thesis I wanted to investigate the regulation of the Rad53 kinase activity, which, in *S. cerevisiae*, is essential to respond to DNA lesions and replication stress (Pellicioli et al., 1999; Sun et al., 1996). It is known that the apical kinases Mec1 and Tel1 phosphorylate Rad53 which, in turn, undergoes extensive autophosphorylation (Pellicioli and Foiani, 2005). Therefore, Rad53 is phosphorylated at many sites, as a result of in trans phosphorylation and autophosphorylation events as demonstrated by two recent works that mapped through mass spectrometry techniques phosphorylation sites before and after DNA damage (Smolka et al., 2005b; Sweeney et al., 2005). This regulatory mechanism is conserved and, in human cells, Chk2 is activated by ATR and ATM (Bartek et al., 2001). We decided to produce monoclonal antibodies against autophosphorylated and active Rad53. To generate the appropriate immunoreactive reagent, we took advantage of the fact that Rad53 over-produced in *E. coli* cells is extensively autophosphorylated (Gilbert et al., 2001). In figure 1 protein extracts from *E. coli* cells before and after induction with IPTG (3 hours) were separated on a SDS PAGE gel and hybridized with anti Rad53 antibodies (upper panel) or analyzed with an *in situ* kinase assay (lower panel). With this technique proteins blotted on a PVDF membrane are subjected to denaturation and renaturation in presence of hot ATP. The appearance of a pattern of lower mobility shift is suggestive of a strong modification of the protein that we verified to be autophosphorylation with the *in situ* kinase assay (Pellicioli et al., 1999). We purified from bacterial cells a full-length

version of Rad53 on a Ni-TA resin column, which was then used for mice immunization (see experimental procedures in this thesis). In order to find monoclonal antibodies that specifically recognize a certain isoform of Rad53 we screened several hybridoma clones by western blot on extracts from yeast cells treated with different DNA damaging agents. In particular we analyzed extracts from cells treated for 1 hour with 4NQO (2μg/ml), or 3 hours of HU (200mM) or MMS (0.02%). 4NQO is an UV mimicking agent, while HU is causing the inhibition of ribonucleotide-reductase complex (RNR) that results in the arrest DNA replication progression; MMS is an alkylating agent that can induce double strand breaks (DSBs). In all these conditions Rad53 is known to undergo to extensive phosphorylation. Indeed, as shown in figure 2, we were able to find several specific monoclonal antibodies that we divided in different classes on the basis of their immunoreactivity and two of them (clones EL7 and F9) were selected for further characterization. In this figure it is also reported the result of ELISA and test sandwich performed by monoclonal antibodies facility in my institute (for details see experimental procedures). In particular, the EL7 monoclonal antibody (Mab) recognizes a broad range of Rad53 isoforms, while F9 Mabs specifically recognize Rad53 isoforms that are found in wild type cells experiencing DNA damage. This kind of analysis suggested that F9 Mab selectively reacts with autophosphorylated and active Rad53 as we can conclude by comparing a western blot with F9 and an *in situ* kinase assay on the same extracts (see also figure 7).

Some clones (like EI4 or Do1) seem to recognize specifically the form of phosphorylated Rad53 that is produced after 4NQO treatment since they recognize only the unphosphorylated band in untreated and in HU or MMS conditions. This result suggests that while the treatment with 4NQO results in the phosphorylation of all the protein present in the extract, treatment with HU or MMS for 3 hours results in the phosphorylation of only a part of the total protein presents in the cell and this kind of modification seems to be different from the one caused by exposition to 4NQO. This observation is consistent with

the observation that cells exposed to different DNA damaging agents show a different pattern of Rad53 phosphorylation (Pellicioli and Foiani, 2005). Alternatively, the portion of unphosphorylated protein reflects the turnover of the protein between phosphorylated and dephosphorylated state. This unphosphorylated protein could otherwise result from some kind of adaptation or recovery. Indeed, in our laboratory, it has been demonstrated that cells are able to adapt to the presence to HU, as they switch off the checkpoint signal and reenter the cell cycle even if HU is still present. Cells arrested in G1 and released in medium containing 50mM hydroxyurea slow down replication progression as a consequences of S-phase checkpoint activation. Nevertheless when S-phase is completed cells switch off the checkpoint signal and progress to G2.When the cells reach the second S-phase they activate again the checkpoint demonstrating that the cells are still able to sense the presence of DNA replication stressing agent (Rache Jossen, unpublished observations).

The phenomenon of adaptation to DNA damage was already described in yeast in cells exposed to an irreparable DSB while the process that leads to switch off checkpoint signal after the removal of DNA damage and the re-entering in cell cycle is commonly known as recovery (for a review see (Harrison and Haber, 2006)).

Other clones instead seem to recognize more than one band in 4NQO and also in untreated conditions, see for example the clone CI4. These antibodies are of particular interest because they could demonstrate the basal activity of Rad53. Further investigations are required to unravel the mechanisms of activation and inactivation of the protein after DNA damage, during checkpoint maintenance and checkpoint inactivation as well as in untreated conditions.

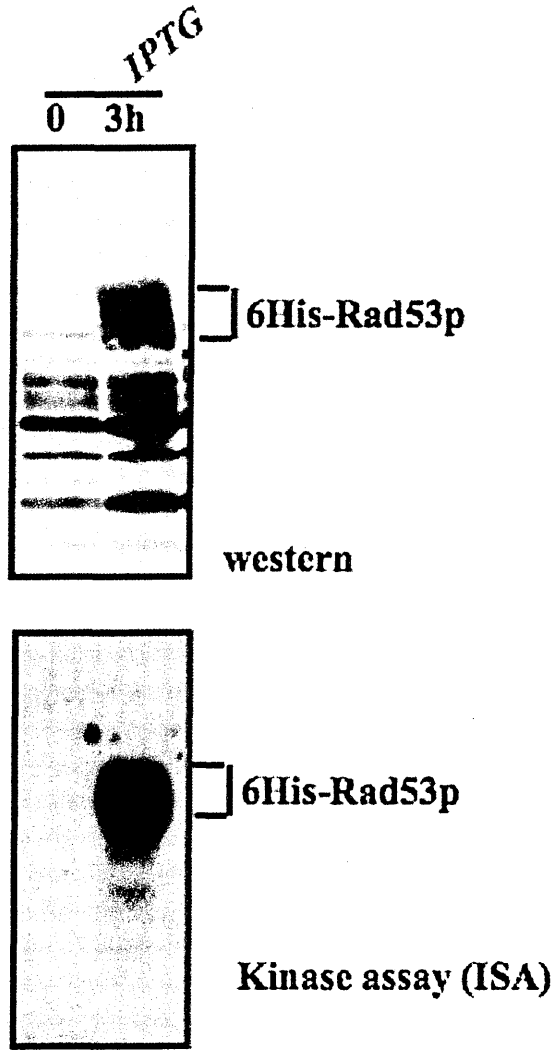


Figure1 .Expression of scRad53 in *E.coli*.

Western blot analisys of Rad53 protein expressed in *E.coli* (upper panel) and *in situ* kinase assay on the same extracts (lower panel). A 25 μg aliquot of total protein was prepared and analysed by ISA or western blotting.

Figure2.Screening for specific monoclonal antibodies.

The name of specific clone is indicated. The results of ELISA and sandwich tests performed by monoclonal antibodies facility are also reported. The panel is completed by western blotting analysis of protein extract of wild type strain. L: exponentially growing conditions, N: 1 hour 4NQO 2µg/ml, H: 3 hours hydroxyurea 200mM, M:3 hours methyl metane sulphonate 0,02%. A 25 µg aliquot of total protein was prepared and loaded on 10% SDS gel

Production of Rad53 mutants on putative ATM/ATR sites

At the beginning of the project, we aimed to produce new phosphomutants of Rad53 in order to unravel the mechanisms of activation and inactivation of this protein. From the analysis of the primary sequence of the protein we identified two putative *consensus* sites for phosphorylation by ATM/ATR-like proteins (Tanaka et al., 2001). Indeed it has been shown that the *consensus* site for PIKK-like kinases coincides with SQ/TQ motifs (Kim et al., 1999). In particular we identified Threonines 8 and 15 in the *N-terminus* of the protein on the basis of their context and their conservation throughout evolution. Through a site directed mutagenesis strategy (see experimental procedures) we produced a series of mutants substituting each single threonine with Alanine (T8A, T15A) in order to mimic the unphosphorylatable form of the protein or with Aspartate (T8D, T15D) in order to mimic the phosphorylated form of the site by introducing a negatively charged aminoacid.

We decided to analyze the effect of each single substitution on the checkpoint functions of Rad53. In particular I analyzed the ability of the protein to undergo to a mobility shift after DNA damage, since phosphorylation of the protein and activation of its kinase activity is always associated with DNA insults (Pellicioli and Foiani, 2005). To this purpose, cells carrying each single mutation were grown in YPD and subsequently treated for 1 hour with 4NQO (2μg/ml). The result of such analysis is reported in figure 3. None of the mutants showed an evident deficiency in the activation of the protein as detected by assessing the mobility shift of mutant proteins in a polyacrilammide gel. Moreover I analyzed the sensitivity of the strains carrying the mutations to hydroxiurea (HU), by plating serial dilutions of cultures of wild type and mutant strains on plates containing increasing amounts of HU (figure 4). None of the single mutants showed any particular sensitivity to this kind of treatment. From these data we concluded that probably these sites can

compensate one for the absence of the other and that the effect on the function of the protein may be evident only on the double mutant. This result is consistent with a paper published afterward; the authors were able to show that a mutant protein of Rad53 carrying T5, T8, T12 and T15 all mutated to Alanine renders the strain sensitive to HU treatment in plate while the re-introduction of one individual Threonine restores viability suggesting that these Threonines are functionally redundant (Bartek et al., 2001; Lee et al., 2003b).

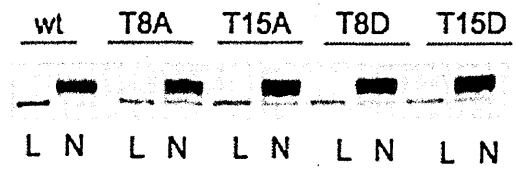


Figure 3. Analisiys of Rad53 alleles on putative ATM/ATR phosphorylation sites. Logarithmically growing cells were treated for 1 hour with 2μg/ml 4NQO (N). A 25μg aliquot of total protein extract was loaded on 10% polyacrilammide gel and hybridized with EL7 Mabs.

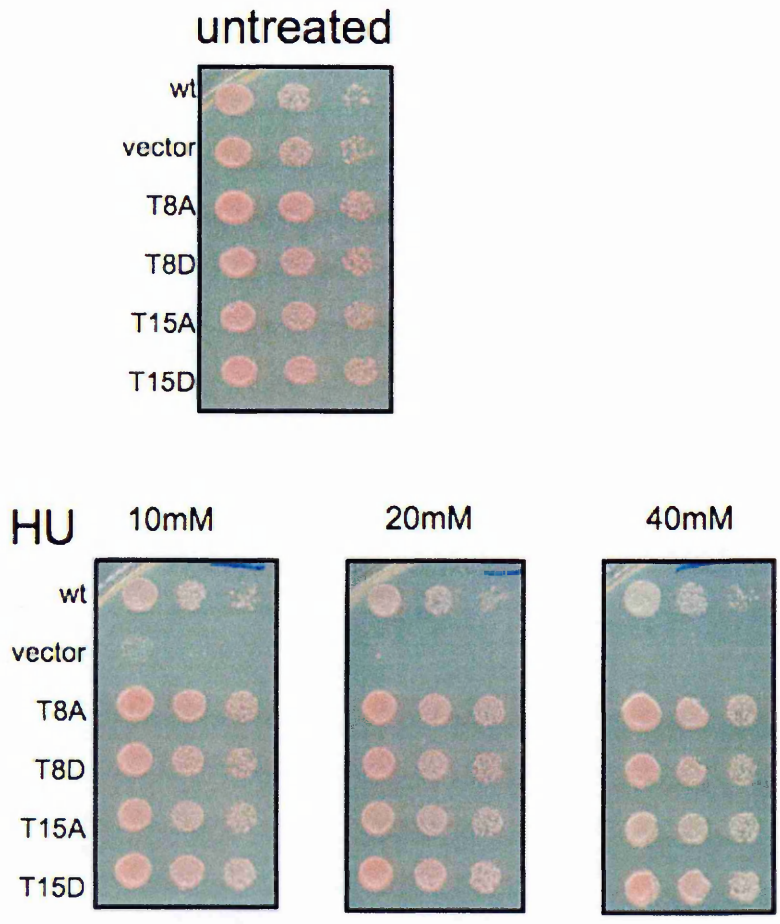


Figure 4. Analisys of HU sensitivity of mutants in putative ATM/ATR phosphorylation sites.

Cells were counted at the same concentration and diluted with a ratio 1:10 before plating the same amount on plates containing HU at increasing concentrations. As a control we used a strain carrying wild type version of the protein as well as a strain lacking *RAD53* gene.

Production of Rad53 mutants at putative autophosphorylation sites

In many protein kinases the kinase domain is target for phosphorylation events that are able to modulate the activity of the protein. In particular some kinases are known to contain a sub-domain, the activation loop or T-loop, that is target for regulation (Nolen et al., 2004). This subdomain is delimited by DFG and APE motifs and often includes Threonine or Serine residues that are target for phosphorylation. In 1996 Johnson and co-workers showed that all kinases regulated through activation segment phosphorylation have a conserved arginine preceding the conserved catalytic aspartate in the catalytic loop and for this reason are known as RD kinases. It has been proposed that phosphorylation of residues in the T-loop have the role of introducing negative charges that counteract the positive charge of the arginine and other aminoacids in the catalytic region contributing thus to the correct conformation of catalytic and substrate-interacting domains. This idea is further supported by two observations: kinases that do not posses the arginine before the catalytic aspartic, are not phosphorylated in the T-loop, and RD-box containing kinases that are not phosphorylated in the T-loop posses negatively charged aminoacids that could predispose the kinase in a constitutive active state. The phosphorylation events in the T-loop can produce different effects on the protein structure. Indeed it has been shown that in some cases this modification produces a conformational change that can lead to modification of interaction partners, exposure of particular surfaces, of the protein or opening of the catalytic domain, thus facilitating the interaction with the substrate (Johnson et al., 1996). From the analysis of the primary sequence of Rad53, we were able to identify DFG and APE motif as well as RD motif in the catalytic core of the protein. This analysis suggested that the protein kinase could be regulated through phosphorylation in the T-loop domain.

We decided to analyze the sequence of the kinase domain of the protein, by aligning the primary sequences from Rad53, Chk2 and Cds1with the Clustalw program available on-line. We found two threonine residues well conserved from yeast to human (figure5) and we speculated for these two residues a possible role in the regulation of the protein. These two residues are also conserved in a downstream factor of the checkpoint response, Dun1, that share some common structural characteristics with Rad53. Previously, it was shown that these two residues are sites for autophosphorylation in human (Lee and Chung, 2001) and furthermore one of them, T354 has been recently identified as site for autophosphorylation in yeast (Sweeney et al., 2005). To address the possible roles of these sites in regulating the kinase activity of the protein, we took advantage of a site directed mutagenesis to produce single or double mutants of threonine T354 and T358 to alanine and or aspartate. We produced a series of mutants that were subsequently analyzed: T354A, T358A, T354D, T358D, T354AT358A, T354DT358D.

Single mutations

Rad53 is an essential gene and its kinase activity is required for viability since mutations that strongly reduce the kinase activity of the protein result in cell death (Fay et al., 1997). The essentiality of the gene is suppressed by deletion of RNR-complex inhibitor Sml1 (Chabes et al., 2003; Elledge et al., 1993); for these reasons we decide to start analyzing the phenotypes of each mutant by introducing the mutated version of the gene cloned in a plasmid under the genomic promoter in a strain lacking both RAD53 and SML1 genes. As a control we used the same strain transformed with an empty vector.

We started analyzing the single mutants performing in plate assays to test viability of the strains carrying the mutation in presence of hidroxyurea (HU) or methyl-metane sulphonate (MMS).

Logarithmically growing cells were counted and diluted at the same concentration; serial dilution were than plated on medium containing 2.5mM, 5mM, 10mM, 20mM, 40mM HU

or 0.001%, 0.0015%, 0.003%, 0.006%, 0.008% of MMS.

In figure 6 it is possible to note that the *rad53-T358D* mutant shows the same sensitivity to HU of a strain lacking *RAD53* gene. The mutation of T354 to Alanine or Aspartate affects viability on HU only at higher doses (40mM), while mutation of T358 to Alanine results in an intermediate phenotype.

When the cells were exposed to MMS, we noticed that only T358D was highly sensitive while the other mutants were only slightly affected.

This result suggests that T358D mimics the absence of the protein and this observation leads to the idea that phosphorylation of T358 could be required to switch off the catalytic activity of the protein. This residue could be dispensable to activate the protein since T358A is less sensitive to HU and MMS than T358D or the empty vector. Mutation of T354 to Alanine results in the same sensitivity to HU or MMS as mutation to Aspartate and the sensitivity is clear only at higher doses, perhaps suggesting the redundant role of T354 respect to T358.

We decided to perform a checkpoint assay on the single mutants to better address the requirement for each single site in checkpoint functions of the kinase. To this purpose we arrested logarithmically growing cells with α-factor (G1) and we released them in a medium containing 0.02% of MMS. At each time point, as shown in figure 7A, we took samples for FACS analysis, to address the cell cycle phase. At the corresponding time points we plated on YPD medium the same amount of cells for treated culture or untreated culture. Comparing the number of colonies formed after tree days of growth at 28 degrees, we built up a graph to assess the viability after MMS treatment. The FACS analysis shows that while the wild type strains is able to slow down replication progression in presence of MMS (after 3 hours wild type cells have not reached G2), all the mutants behave like the empty vector. As shown in figure 7B, the T358D behaves like the control with the empty vector with respect to viability while the other mutants are less sensitive. These results are in agreement with the previous experiment and suggest that T358 strain is the most

sensitive to a transient exposure to a replication stress. This observation suggests that mutation of one of the two Threonines placed in the T-loop domain of Rad53 causes defect in fork restarting after exposure to MMS.

Analysis of the double mutants

In a recent work it has been reported that T354 of Rad53 is an autophosphorylation sites (Sweeney et al., 2005); in this work the authors identified by mass spectrometry the phosphorylation sites of a strain carrying a wild type version of RAD53 and a strain carrying kinase dead allele of RAD53 treated or not with 4NQO. By the compairison of sites detected in wild type and kinase dead allele they identified T354 as an autophosphorylation site. Since in our hands the mutation of T358 site showed a stronger phenotype than mutation of T354 we decided to analyze the double mutants. To test the effect of the introduction of the double mutation in a more physiological condition, we sub-cloned the mutant genes in an integrative plasmid (derived from pCH8, see experimental procedures) and we substitute the genomic RAD53; in these strains SML1 gene is deleted. We then analyzed the level and the activity of the mutant Rad53 proteins in cells growing in normal conditions or experiencing DNA damage or replication stress (figure 8). Cells of strains rad53-AA and rad53-DD were exposed to 4-nitroquinoline-oxide (4-NQO) or to hydroxyurea (HU), or to methyl metane sulphonate (MMS). As controls we used wild type cells and cells carrying a kinase-defective version of RAD53 (rad53-K227A). To test the level and modification of Rad53 protein in the extracts of the indicated strains, we took advantage of the newly produced monoclonal antibodies. In the figure 7 we can observe the results obtained by monitoring Rad53 on western blots probed with EL7 or F9. Moreover, to test directly the Rad53 kinase activity, we measured Rad53 autophosphorylation in an *in situ* kinase assay developed in our laboratory (Pellicioli et al., 1999) and performed on the same protein extracts analyzed by western blotting. We found

that, although the mutagenized versions of Rad53 protein have been phosphorylated at an intermediate level, both the AA and the DD mutations strongly prevent the autophosphorylation of Rad53 protein in response to DNA damaging agents, thus recapitulating what we previously found in the kinase-defective rad53-K227A allele.

We performed in plate viability assays to assess the sensitivity of the double mutants to HU or MMS. As shown in figure 9 the double mutant DD is more sensitive to HU than K227A, while AA shows an intermediate phenotype. This result recapitulates what found with the mutation of T358 to Alanine or Aspartate suggesting that the mutation of the single residues T358 is sufficient to abolish the kinase activity and the checkpoint function of Rad53.

Taken together our results suggest that T354 and T358 residues located in the Rad53 activation loop influence its kinase activity similarly to what was shown for Chk2 and Cds1 (Lee and Chung, 2001; Xu et al., 2006). Moreover our results rule out the possibility that the Rad53DD protein variant mimics a constitutively active Rad53 protein as demonstrated by the absence of kinase activity in the *in situ* kinase assay. This result was also suggested by the expression of the mutant versions AA and DD in *E. coli* figure 10; indeed unlikely the wild type protein these two versions do not undergo to autophosphorylation. Nevertheless we cannot exclude the possibility that these proteins are active toward specific substrates since mutation of this two residue could in theory prevent insertion of 32p ATP because if they are the unique sites for autophosphorylation. This hypothesis seems to be unlikely given the amount of autophosphorylation sites that was suggested by the findings of Sweeney and co-workers.

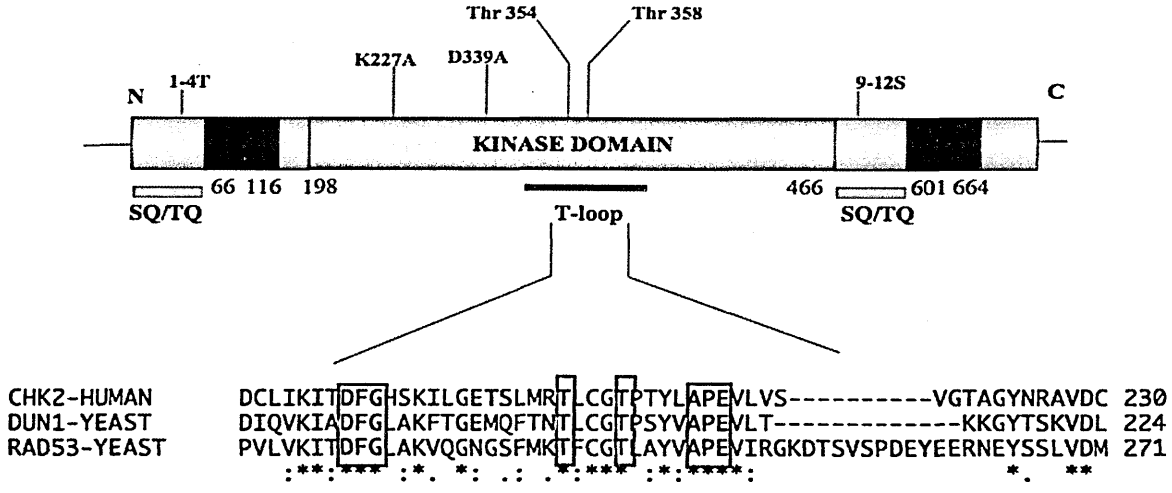


Figure 5. Clustalw alignement of primary sequence of activation loop of yRad53, hChk2 and yDun1.

The primary sequences of the kinase domains of yeast Rad53, yeast Dun1 and human Chk2, were used as query for clustalw program at the www.ebi.ac.uk/Tools/clustalw2/index.html web site.

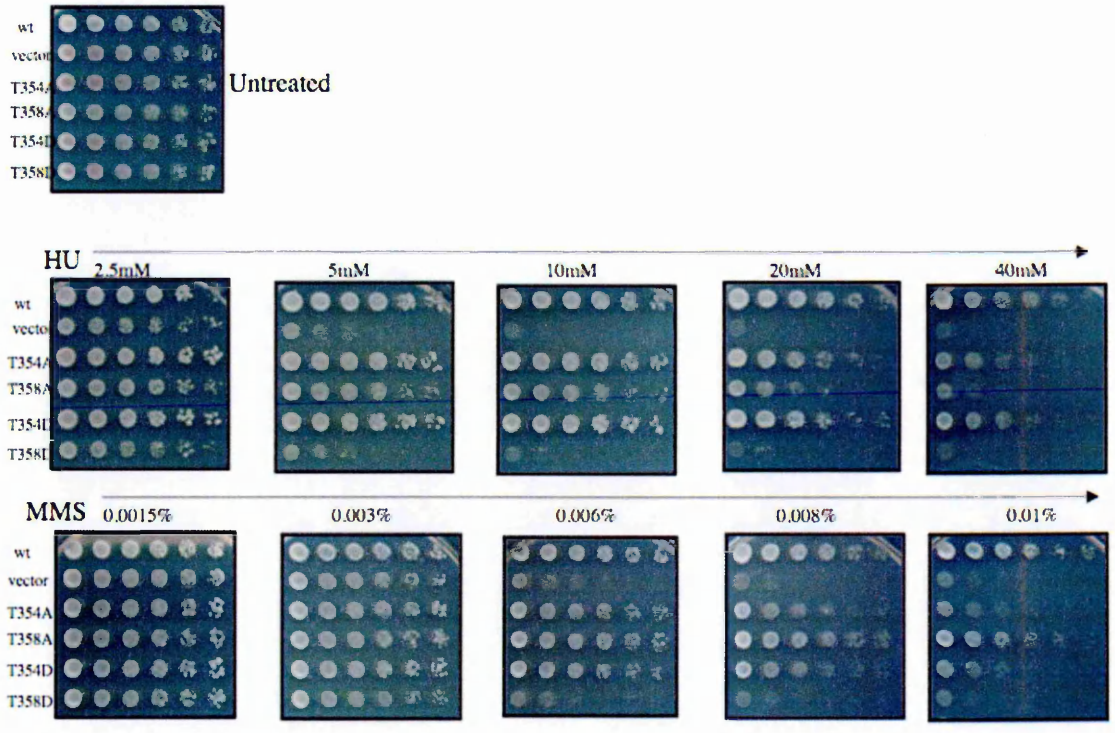


Figure 6. HU and MMS sensitivity of T-loop mutated *rad53* alleles.

Drop test analysis of serial 5-fold dilutions of Δrad53Δsml1 carrying an empty vector, a wild type, or mutated version of RAD53 gene. Cells were plated on agar plates woth or without HU and MMS at the indicated concentrations. The plates were incubated 3 days at 28°C

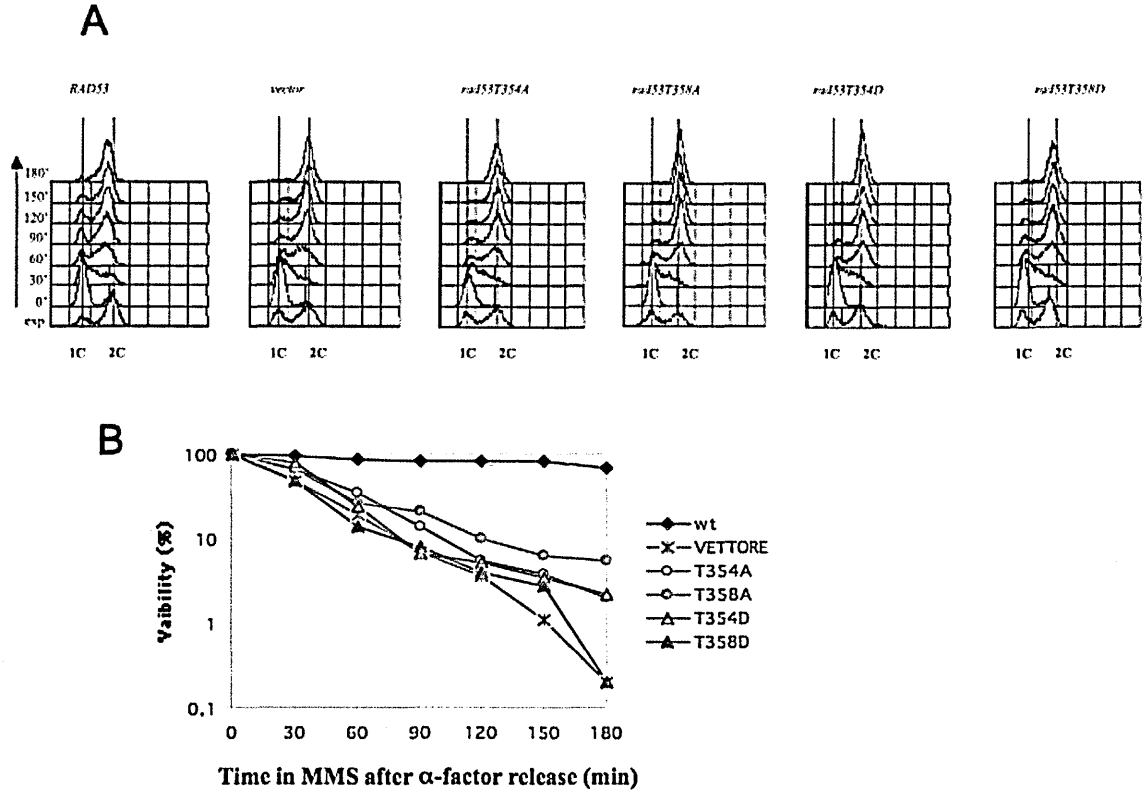


Figure 7. S-phase checkpoint in T-loop mutants.

A. FACS analisys of wild type, control empty vector and T354A, T358A, T354D, T358D rad53 mutants, arrested in α-factor and released in presence of 0.02% MMS. B. Viability of indicated strains as measured by plating cells at the indicated time points on YPD plates.

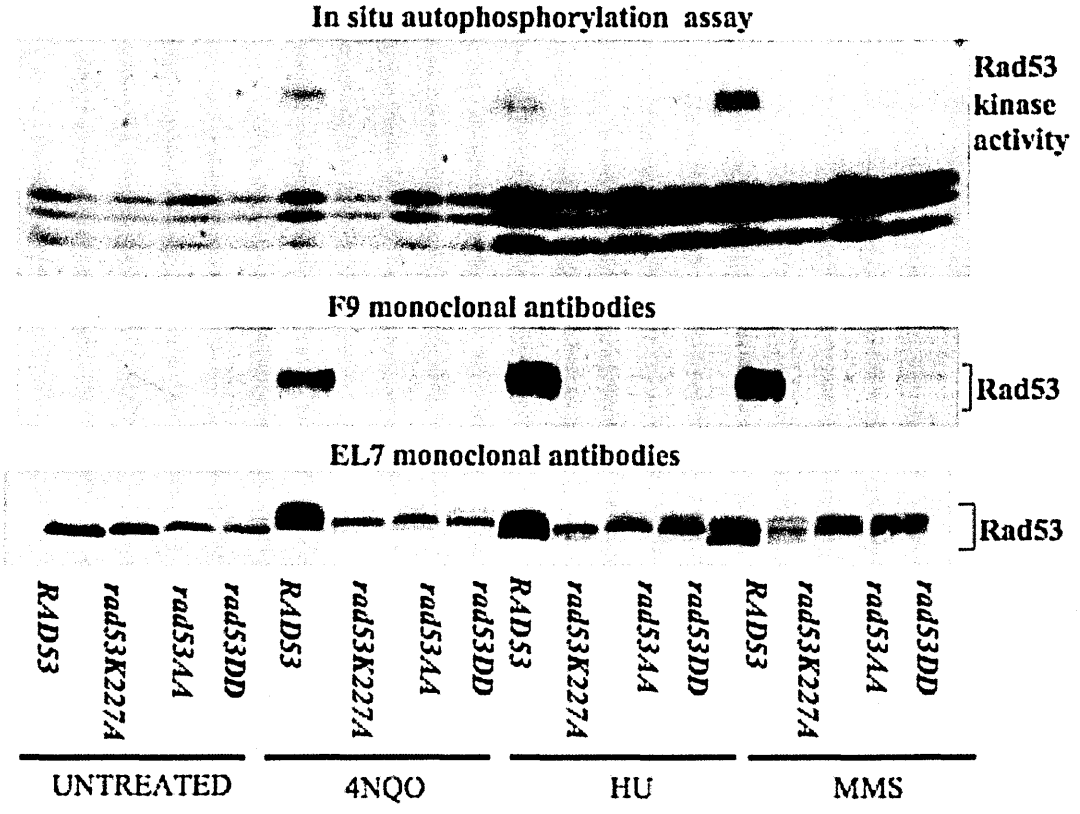


Figure 8. Levels and phosphorylation state of Rad53 protein in various *rad53* alleles

Exponentially growing (untreated) cells of isogenic strains W303 (*RAD53*), CY2034 (*rad53*-K227A), CY5926 (*rad53*-AA) or CY6166 (*rad53*-DD) were treated for 3 hours with 0.2 M HU, for 3 hours with 0.02 % MMS or for 1 hour with 2 μg/ml 4NQO. A 25 μg aliquot of total protein was prepared from the indicated strains and analysed by ISA or western blotting using monoclonal antibodies. F9 antibodies were used to test autophosphorylated and active state of Rad53

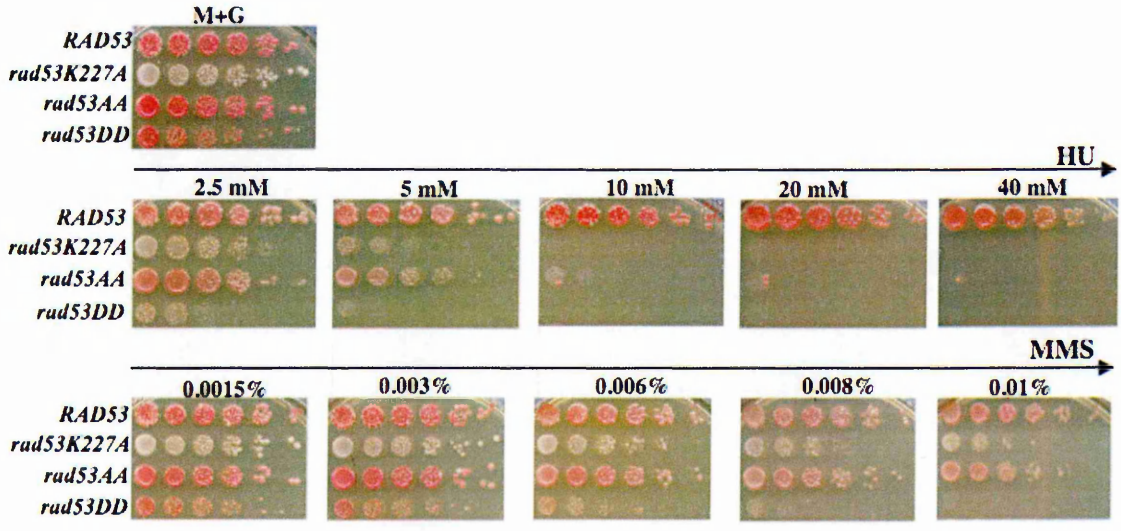


Figure 9. In plate sensitivity assay of T-loop mutants

Serial dilution of cultures of indicated strains were plated on YPD agar plates containing the indicated concentreation of HU and MMS. After three days of incubationat 28 degrees plates were analyzed.

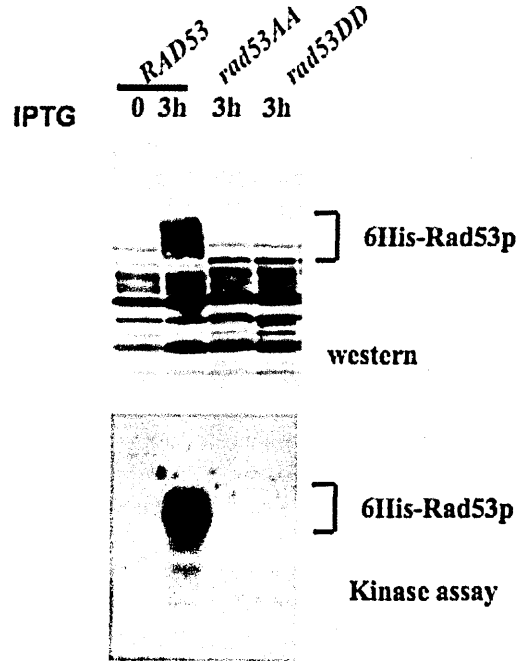


Figure 10 .Expression of scRad53 wild type or -AA -DD mutants in *E.coli.*

Western blot analisys of Rad53 protein expressed in *E.coli* (upper panel) and *in situ* kinase assay on the same extracts (lower panel). A 25 μg aliquot of total protein was prepared and analysed by ISA or western blotting.

T354 and T358 are bona fide autophosphorylation sites.

In order to understand if the two Threonine T354 and T358 are real sites for autophosphorylation we decided to produce two new alleles of the protein, T354ST358S and T354ET358E with the idea to introduce back the possibility to phosphorylate the T-loop with the Serine residue or in order to mimic in a different manner the phosphorylated state by introducing a charged aminoacid (the Glutammic acid) with a different shape with respect to Aspartate.

Cells deleted for *RAD53* and *SML1* genes carrying the wild type or mutated gene on a plasmid and the control empty vector were counted at the same concentration and plated in serial dilutions on plates containing growing amount of HU or MMS. As a control the same cultures were plated on agar plates without any drug. After 3 days of growing at 28°C, the plates were compared.

In figure 11 we can observe that *rad53-SS* mutant shows the same sensitivity to HU as the wild type, while the mutation to Glutamate has as severe consequences as the mutation to Aspartate. Analyzing the plates containing MMS we can observe that *rad53-SS* mutation is able to confer to the cells the same viability as the wild type protein. Double *rad53-EE* mutation instead, sensitizes the cells to the presence of an alkylating agent at the same level as *rad53-DD* mutation. This result suggests that the possibility to phosphorylate the T-loop of the kinase its sufficient to allow survival in the presence of chemical agents that cause replication stresses.

To better understand the phenotype of *rad53-SS* and *rad53-EE* mutations we decided to perform a complementation assay (figure 12) in which we reintroduced in AA or DD mutants strains the wild type protein or the double SS protein and tested the sensitivity to high dose of hydroxyurea (40mM). The cells were plated at the same concentration on

plates without any drug or with HU. After three days of growth a 28°C the plates were compared.

I noticed that at this concentration the wild type protein was able to fully restore the viability of both mutants while the double SS version could only partially do it. This result suggests that in presence of a mutated form of *RAD53*, either AA or DD, the serine variant is not able to function properly or with the same efficiency as the wild type. This result could be suggestive of a stringent specificity of the kinase that would prefer Threonine residues to Serine residues as a substrate of phosphorylation and that this kinase activity is required only at higher doses. Alternatively SS mutation in the T-loop of the protein could interfere with the conformation of the protein influencing homo- and/or hetero-interactions.

This experiment further confirmed that all these mutation are recessive supporting the idea the all of them represent loss of function mutations.

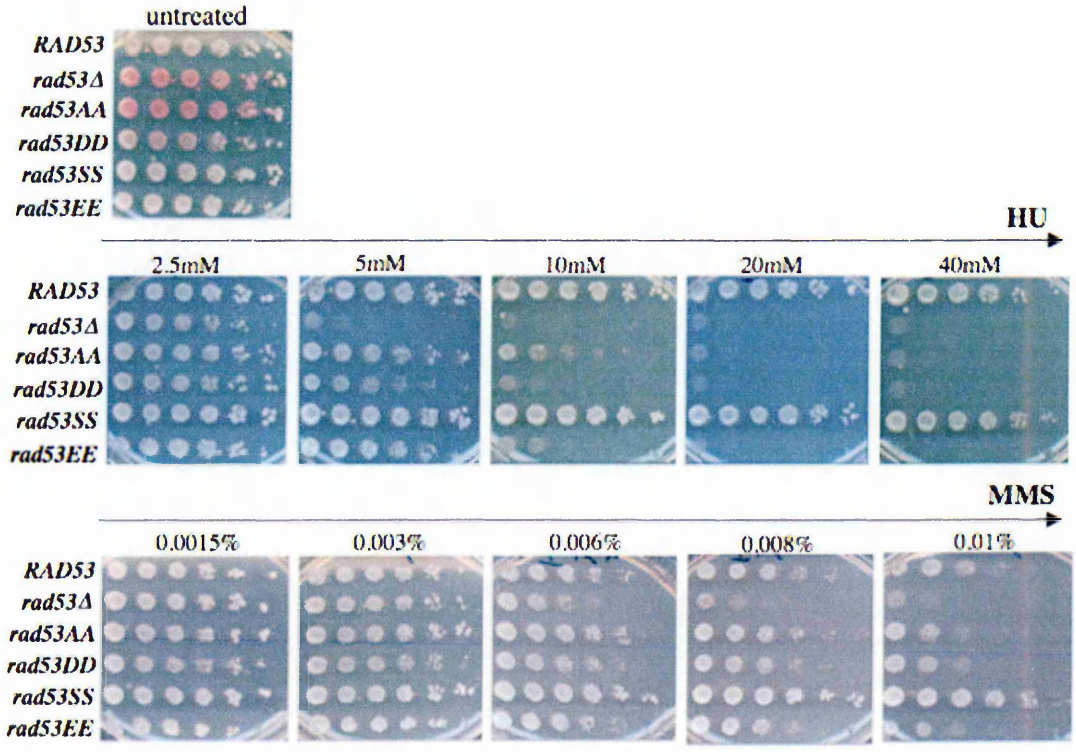


Figure 11. Analisys of HU and MMS sensitivity of mutants in putative autophosphorylation sites.

Cells were counted at the same concentration and diluted with a ratio 1:6 before plating the same amount on plates containing HU or MMS at growing concentrations. As a control we used a strain carrying wild type version of the protein as well as a strain lacking *RAD53* gene.

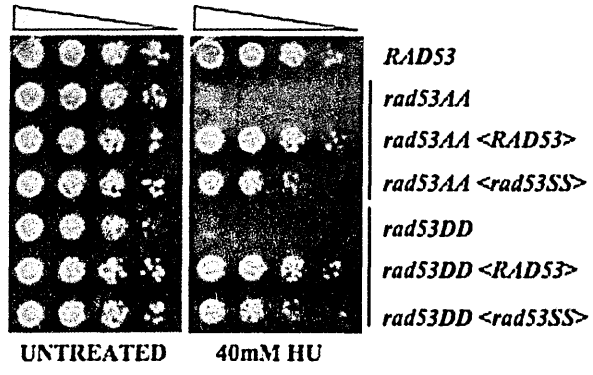


Figure 12. HU sensitivity of T-loop mutated *rad53* alleles.

Drop test analysis of serial 5-fold dilutions of the indicated strains plated on YPD and HU plates, incubated 3 days at 28°C.

Dominant negative phenotype of AA and DD rad53 mutants

The over-expression of *rad53*-D339A kinase-defective allele, have been previously described to cause a dominant checkpoint-defective phenotype (Pellicioli et al., 1999; Sun et al., 1996). To investigate this phenotype also in *rad53*-AA and *rad53*-DD alleles, we cloned the mutagenized version of *RAD53* gene in an integrative vector under the control of the inducible *GAL1* promoter (see experimental procedures). The plasmids have then been introduced into a wild type strain carrying the tagged *RAD9*-9myc and *DUN1*-3HA genes; *RAD9* and *DUN1* act in the checkpoint signal transduction pathway upstream or downstream of Rad53, respectively (Zhou and Elledge, 2000). When cells of the indicated derivative strains were allowed to enter S phase in the presence of MMS to activate the checkpoint and in the presence of galactose (2%) to induce the expression of the *rad53* alleles, cells progressed through S phase more quickly than control cells grown in MMS and raffinose (figure 13). Progression through the cell cycle was accompanied by a concomitant decrease in the endogenous Rad53 activity, measured by the *in situ* kinase assay (figure 13 upper panel), and by the dephosphorylation of the Dun1 protein, tested by western blotting (figure13 lower panel). Phosphorylation of Rad9 (figure 13 middle panel) was not affected by the overexpression of the kinase-defective *rad53* alleles, as expected for a factor playing a role upstream Rad53 in signal transduction pathway and accordingly to what previously described (Lee et al., 2003b). The results shown in figure 13 suggest that phosphorylation of T354 and T358 in Rad53 kinase plays crucial roles for checkpoint activation and maintenance during a perturbed S phase.

To further confirm the dominant negative phenotypes of *rad53-AA* and *rad53-DD* mutants, we repeated the in plate assay to measure the sensitivity to the presence of DNA replication stresses (HU and MMS). As it is shown in figure 14 the over-expression of

each single mutant greatly affect the viability in presence of even low amount of HU. The presence of these mutations confers sensitivity to MMS too. In both cases the effect seems to be stronger than in the presence of the over-expressed kinase dead *rad53-D339A*. These results suggest that the over-expression of a mutant version of *RAD53* could titrate the signal coming from upstream factors or affect the formation of homodimers Rad53, or alternatively affect the formation of heterodimers Rad53-Dun1. Accordingly with wath previously suggested (Pellicioli et al 1999) this finding suggests that a functional checkpoint probably requires a threshold level of Rad53 Kinase and that Rad53 activity is required for checkpoint maintenance and not only for its activation.

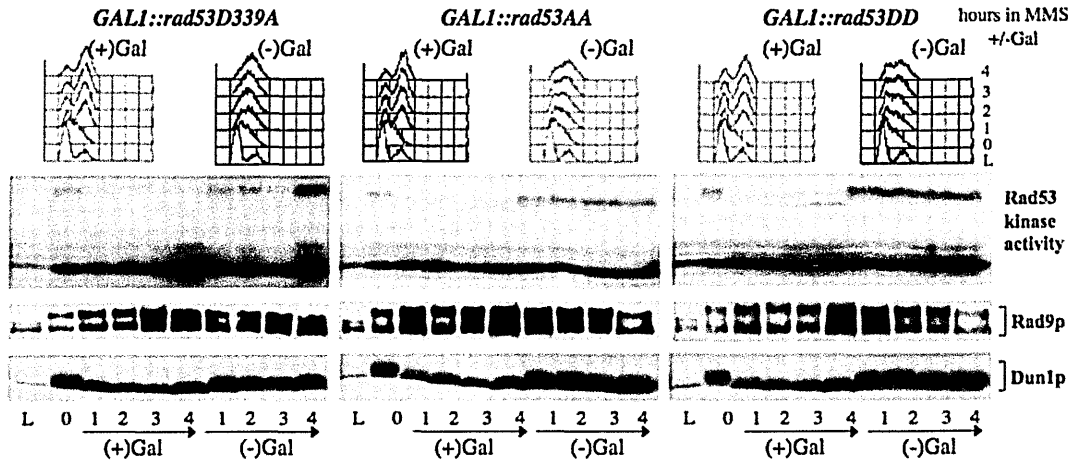


Figure 13. Phosphosites mutants of Rad53 T-loop are dominant negative checkpoint defective alleles.

Exponentially growing cultures of the strains CY5977 (GAL1::rad53-D339A), CY5978 (GAL1::rad53-AA) and CY5979(GAL1::rad53-DD) were grown in 2% raffinose and treated for 3 hours with 0.2 M HU (0). Galactose (2%) was then added to half of the culture, while the other half was maintained in raffinose. Samples were taken at the times indicated and analysed by FACS. A 25 mg aliquot of total protein was prepared from the indicated strains and analysed by ISA to monitor Rad53 activity or by western blotting using anti-HA antibodies to test Rad9-HA and Dun1-HA proteins.

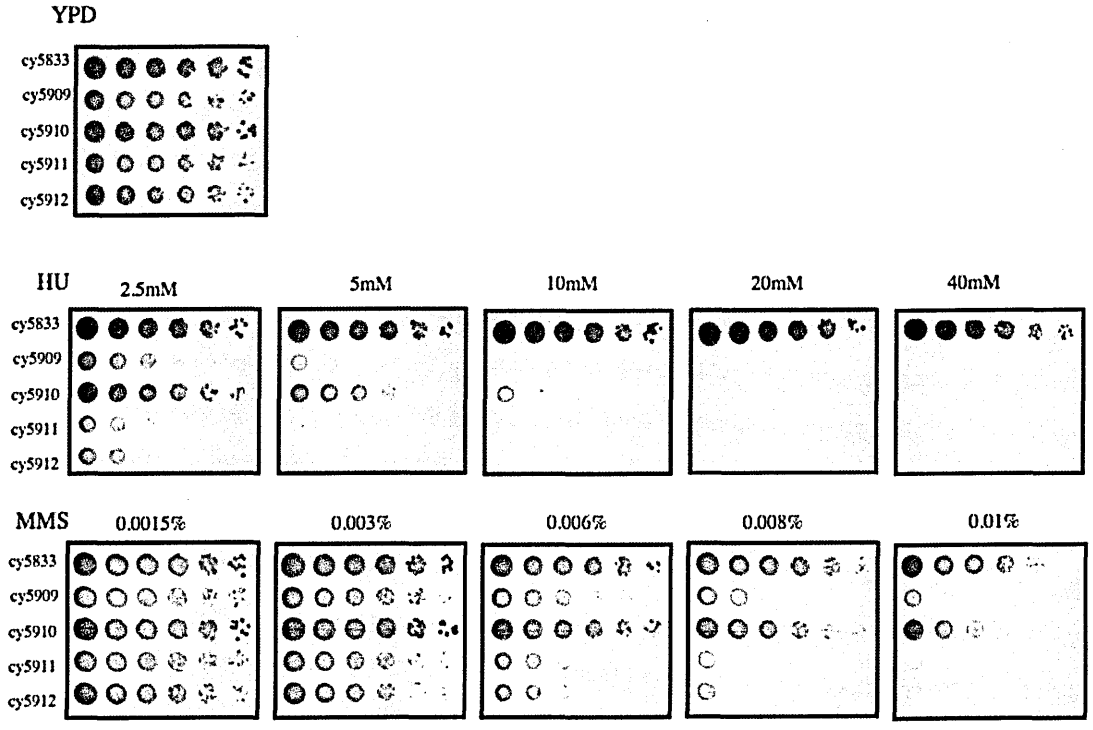


Figure 14. Over-expression of T-loop mutants in damaging conditions.

Cells were counted and diluted 1:5. Drops from cultures of indicated strains cy5833 (wt), cy5909 (*GALRAD53*), cy 5910 (*GALrad53D339A)*, cy5911 (*GALrad53AA*), cy5912 (*GALrad53DD*)were plated on YPD plates containing growing amount of HU and MMS or on YPD plates withouth any drug.

De-repression of checkpoint response in rad53-DD and –TD alleles

During the characterization of the mutated *rad53* alleles, we noticed that Rad53 is slightly modified in *rad53*-DD cells growing in unperturbed condition (figure 15). We speculated this modification to be the result of the conformational change induced by the introduction of an aminoacid with negative charge within the T-loop domain of the kinase core. This hypothesis was excluded by the observation that modification of the Rad53-DD mutant protein is prevented by *SML1* deletion as we can appreciate in figure 16; nevertheless, deletion of *SML1* gene does not rescue the sensitivity to hydroxyurea due to the presence of DD mutation (figure 17A and B). We also found (figure 15) the same level of modification of Rad53 protein in the cells carrying the single mutation *rad53*-TD, but not in the single *rad53*-DT mutant cells. We wondered if this modification was due to a checkpoint signal active in these mutants even in unperturbed conditions. To address this possibility we decided to treat the cells with caffeine that is known to inhibit ATM/ATR related proteins (Cortez, 2003). As it is shown in figure 18, treatment with caffeine completely abolishes the phosphorylation of *rad53-DD* protein, suggesting that this modification is due to a checkpoint response as a consequence of some kind of DNA lesions. In presence of *rad53-DD* mutation we can speculate that the inability of the protein to work properly is the cause of the accumulation of DNA damage. Alternatively we could argue that this mutation affects the ability of the protein to promote repair processes that deal with physiological DNA damage that goes with normal DNA replication. This seems not to be the case since in absence of *SML1* the DNA damage response is not active while the inability of the protein to work properly should be identical as demonstrate by the fact that *rad53-DD sml1*Δ cells are still greatly sensitive to HU treatment (figure 17).

The fact that this treatment abolishes the mobility shift confirms what we concluded from previous experiments (see paragraph "analysis of double mutants" and figure 9) as it excludes the possibility that this mobility shift is due to a constitutive conformational change introduced by the mutation to Aspartate.

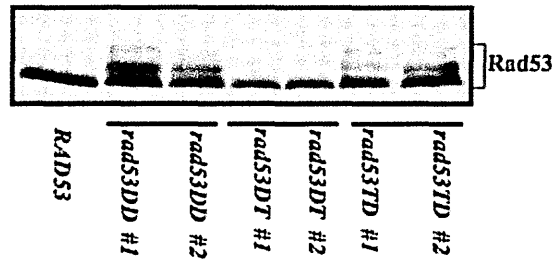


Figure 15. Analysis of rad53 protein in DD mutants and in TD or DT mutants.

A 25 μg aliquot of total protein from cells carrying wild type or mutated version of RAD53 was analyzed by western blotting with EL7 Mab in exponentially growing conditions. The strains are: CY5833 (wild type RAD53), C6166 (rad53-DD), CY6167 (rad53-DD), CY6190 (rad53-DT), CY611 (rad53-DT), CY6274 (rad53-TD), CY6275 (rad53-TD).

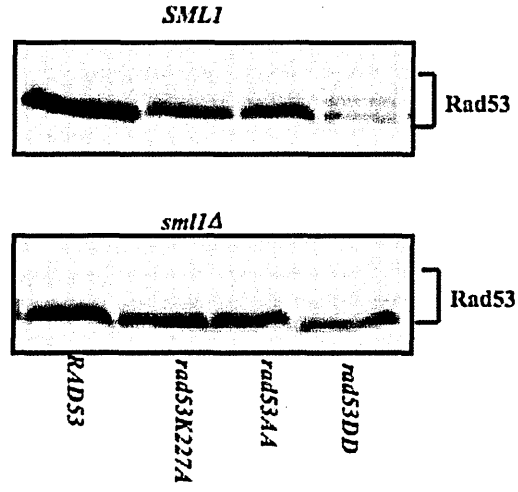


Figure 16. Analysis of *rad53 alleles* in presence or absence of *SML1*.

A 25 μg aliquot from total extract prepared from exponentially growing cells of indicated strains were loaded. Western blot was performed with EL7 Mabs.

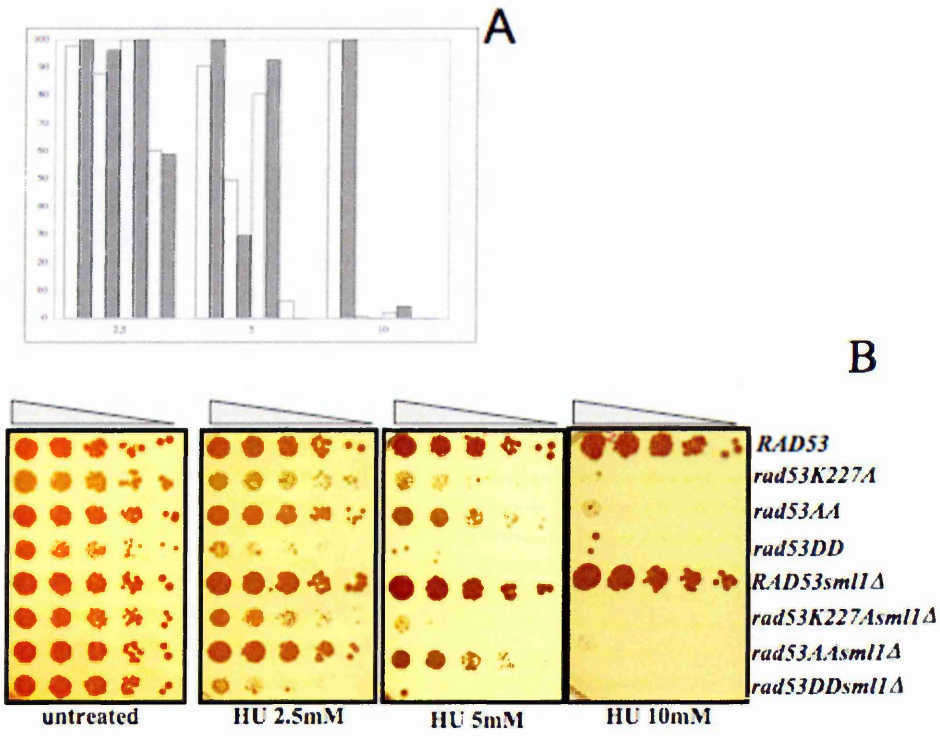


Figura 17. HU sensitivity of T-loop mutated *rad53* alleles.

A quantification and B Drop test analysis of serial 5-fold dilutions of the strains CY5833 (wild type *RAD53*), CY2034 (*rad53*-K227A), CY5926 (*rad53*-AA), CY6166 (*rad53*-DD), CY3146 (*RAD53 sml1*Δ), CY5841 (*rad53*-K227A, *sml1*Δ), CY5923 (*rad53*-AA, *sml1*Δ), CY5924 (*rad53*-DD, *sml1*Δ). Cells were plated on YPD and YPD plus HU at the indicated concentrations. The plates were incubated 3 days at 28°C.

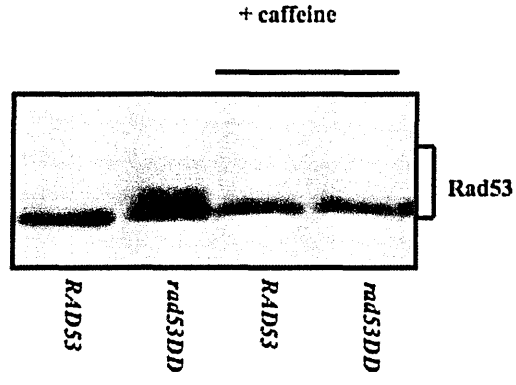


Figure 18. Caffeine treatment abolishes modification of rad53-DD protein.

Samples were taken from exponentially growing cultures of the strains CY5833 (wild type RAD53), CY6166 (rad53-DD), before and after the treatment with 10 mg/ml caffeine 0.5 hr

Lethality vs slow growth in rad53 mutants

During the characterization of the phenotype of the mutants we noticed that that the *rad53*-TD cells show the *SML1*-dependent small colony phenotype observed also in *rad53*-DD cells (Figure 19). This phenotype could be theoretically due to two different but not necessarily alternatives causes: delay through S-phase progression and/or accumulation of events that lead to cell death. Several lines of circumstantial evidence suggest that in addition to its checkpoint function, Rad53p could be also involved in the regulation of temporal order origin firing during chromosomal DNA replication (Santocanale and Diffley, 1998). In addiction, it was shown that Rad53p phosphorylates the Cdc7p/Dbf4p complex in vitro and that this phosphorylation greatly inhibits the kinase activity of Cdc7p/Dbf4p (Kihara et al., 2000). This result suggests that Rad53p controls the initiation of chromosomal DNA replication by regulating the protein kinase activity associated with the Cdc7p/Dbf4p complex. From this data we can speculate a role for Rad53 in "normal" S phase.

In order to verify the S-phase progression rate of *rad53-DD* allele respect to the wild type, we performed an accurate analysis of the cell cycle progression trough fluorescence-associated cell sorting (FACS).

Cells from wild type and *rad53-DD* were arrested in α–factor and released in fresh medium at 20°C to slow down the S-phase progression. The FACS analysis reported in figure 20 shows that the mutant is slightly slower in replicating DNA with respect to the wild type. We judged that the effect on S phase progression was not strong enough to account for the phenotype of small colony formation observed previously, thus we wondered if the difference observed in the growing rate could be due to high lethality in *rad53-DD* mutants. We decided to perform an in plate viability assay.

Unbudded cells were micromanipulated and isolated on plates of YPD and after three days

colony formation was scored. In figure 21 an example of such analysis is reported. We noticed a high lethality in *rad53-DD* mutants and this lethality was suppressed by deletion of *SML1* gene. Approximately half of the cells plated from *rad53-DD* culture didn't give rise to any colony (viable colonies: 52%), while almost all the cells from *rad53-DDsml1Δ* strain gave a viable colony (viable colonies: 92%). The analysis were performed on 120 aploid cells for each strain. We noticed also that rad53-DD cells showed a heterogeneous colony size demonstrating that lethal events arise at different steps or require accumulation through many generations. The high lethality observed in *rad53-DD* mutants is probably responsible for the small colony phenotype; this observation together with the observation that rad53-DD show a modification of the protein dependent on apical kinases let us to speculate that the observed lethality was due to a kind of DNA damage caused by the presence of *rad53-DD* mutation. This lethality could be due to the presence of an irreparable DNA damage or to a permanent checkpoint arrest. The absence of *SML1* is able to rescue the cell lethality.

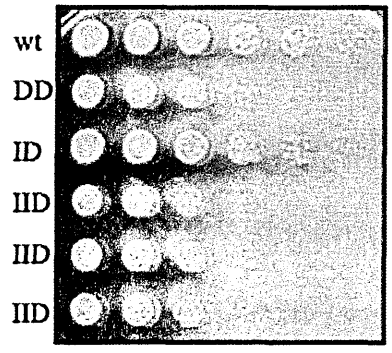


Figure 19. Analysis of growth rate in DD mutants and in TD or DT mutants.

Drop test analysis of serial 5-fold dilutions of the strains CY5833 (wild type *RAD53*), C6166 (*rad53*-DD), CY6167 (*rad53*-DD), CY6190 (*rad53*-DT), CY611 (*rad53*-DT), CY6274 (*rad53*-TD), CY6275 (*rad53*-TD). Cells were plated on YPD and YPD plus HU at the indicated concentrations. The plates were incubated 3 days at 28°C.

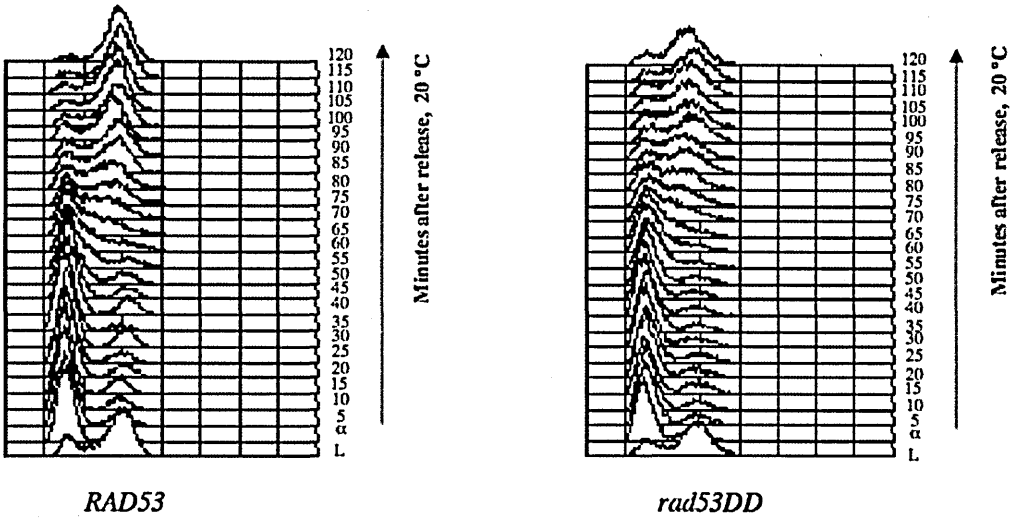


Figure 20. *rad53-DD* S-phase progression.

Cells were arrested in G1 and released in fresh medium to allow S-phase progression. Samples were taken every 5 minutes and analyzed by FACS.

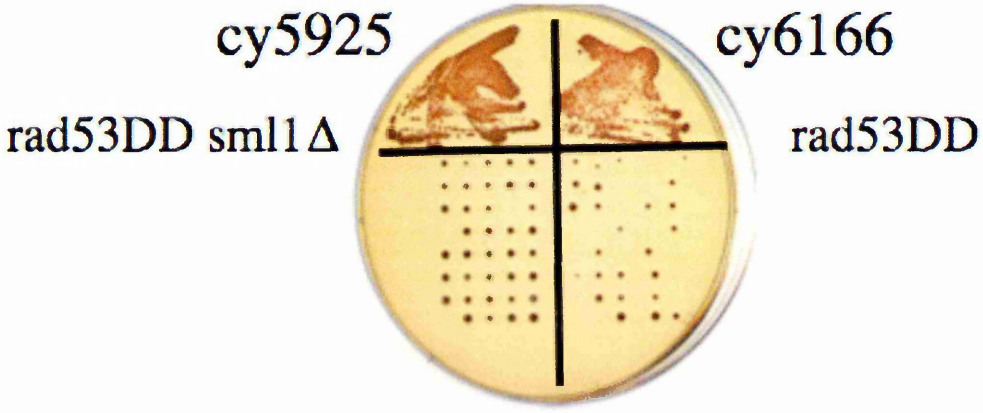


Figure 21. Viability test.

120 aploid cells from cy5925 (rad53-DDsml1Δ) and cy6166 (rad53-DD) were plated as umbudded and incubated at 28°C for three days

Double strand breaks accumulate in rad53-DD

As shown in the previous paragraphs *rad53-DD* mutants are accumulating some kind of DNA lesions that activate the checkpoint response and lead to cell death. We wanted to understand which kind of lesions *rad53-DD* mutation was generating. Since Rad53 has been implicated in fork stability in presence of replication stresses (Lopes et al., 2001; Lucca et al., 2004), we speculated that affecting the kinase activity of Rad53 would result in problems in replicating DNA even in unperturbed conditions. The kinase activity of the protein is indeed required to prevent replication fork collapse in presence of HU (Lopes et al., 2001; Lucca et al., 2004). Furthermore *RAD53* is an essential gene supporting the idea that a basal activity of the protein is required for survival in unperturbed conditions. Problems in replicating DNA would probably generate double strand breaks (DSB) as a consequence of replication fork collapse since the kinase dead allele K227A results in exensive accumulation of ssDNA regions at replicating bubbles in presence of HU (Cotta-Ramusino et al., 2005). The requirement for Rad53 activity in stabilizing arrested replication forks suggests that Rad53 could be required in unperturbed condition in particular regions of the genome, were, physiologically, replication progression slow down. If this hypothesis is true absence of Rad53, would cause problems in replication of particular regions of the genome more than affect the speed of replication of all the genome. A similar role was already described for Mec1 by the study of Cha and co-workers that mapped chromosomal locations that are subjected to double strand breaks in absence of Mec1 activity in unperturbed conditions, namely replication slow zones (Cha and Kleckner, 2002). By this view, DNA replication checkpoint could represent amplifications of functions having a more basic role.

H2A phosphorylation is considered a marker of DNA damage and in particular was identified as a marker for DSBs formation (Downs et al., 2000).

For these reasons we decided to analyze the phosphorylation of S129 residue of histone H2A in unperturbed conditions or after 3 hours of HU treatment (200mM) in wild type *rad53-K227A*, *rad53-AA*, *rad53-DD* mutants (figure 22). In wild type cells H2A is only slightly phosphorylated without HU; this is consistent with what was previously found (Ficarro et al., 2002). H2A indeed is transiently phosphorylated in S-phase (Ficarro et al., 2002). Treatment with HU does not increase the level of phosphorylation of H2A in wild type indicating that this cells are not accumulating DSBs. *rad53-K227A* and *rad53-AA* mutants show no increase in phosphorylation in untreated conditions, while after HU treatment there is a huge phosphorylation of S129, consistent whit the hypothesis that the absence of kinase activity causes replication fork collapse and finally DSBs. As expected following HU treatment DD mutants show hyper-phosphorylation of H2A as we detected for the other kinase dead alleles.

We found that the S129 residue of histone H2A is phosphorylated in *rad53*-DD, but not in *rad53*-DD *sml1*Δ cells growing in unperturbed conditions (figure 23). As such H2A phosphorylation is considered as a marker of DNA lesions (Downs et al., 2000) our results indicate that *rad53*-DD cells accumulate spontaneous DNA breaks during an unperturbed cell cycle, suggesting that the apical Mec1 and Tel1 kinases must be active in *rad53*-DD cells growing in unperturbed conditions (this suggested also by previous experiments). The accumulation of DNA lesions is likely the source of the increased cell lethality and small-colony phenotype observed in *rad53*-DD cells as previously discussed. Since the accumulation of phosphorylated H2A is suppressed by deletion of *SML1*we asked if the presence of RNR-complex inhibitor is causing DNA lesions in *rad53-DD* background. We than analyzed the phosphorylation status of H2A in a dun1Δ strain in which Sml1 protein is not degraded. As shown in figure 24 deletion of DUN1 does not result in accumulation of phosphorylated H2A in untreated condition; this observation suggest that the stability of Sml1 protein is not able per sè to induce DSBs formation. Furthermore we noticed that

H2A was not phosphorylated in treated cells, thus confirming the ability of a Δ*dun1* strain to stabilize replication forks in presence of HU (200mM).

To test the possibility that DSB formation in *rad53-DD* cells was due to the passage through S-phase, I sinchronyzed in G1 cells from wild type and *rad53-DD* and I released them in fresh medium to analyze H2A phosphorylation with the progression through S-phase in unperturbed conditions.

In figure 25 we can notice that DD shows a pronounced S129 phosphorylation with respect to the wild type. Furthermore the phosphorylation appears to be anticipated on time respect to the wild type; this phenomenon is unlikely to be due to the normal S phase phosphorylation in *rad53-DD*, since, as already demonstrated the progression through S phase in DD mutants is slower than the wild type. This experiment suggests that DD mutant is accumulating DSBs during DNA replication.

To address more directly the possibility that the presence of *rad53-DD* was causing double strand breaks formation we performed a PULSE FIELD analysis as described in experimental procedures. Cells of wild type and *rad53DD* strains in logarithmical growth were arrested and fixed in agarose plugs. As a control we used mec1-40 strain that was previously showed to accumulate DSBs during replication when shifted at non-permissive temperature(Cha and Kleckner, 2002). Cells of *mec1-40* stain were arrested in G1 at 25°C and released for three hours at 37°C. After pulse-field gel electrophoresis (PFGE) we labeled the chromosome III with CHA1 probe that is recognizing the CHA1 gene on the chromosome. This technique is called end-labeling Southern and allow the detection of Chromosome species smaller than chromosome three expected in a strain accumulating DSBs. In figure 26 it is possible to note that wild type DNA shows only one specific band corresponding to the chromosome III, while both *mec1-40* and *rad53DD* mutants showed a smear of lower molecular weight that is reflecting fragments from chromosome III.

This result clearly demonstrates that strains carrying the rad53DD mutation accumulate DSB.

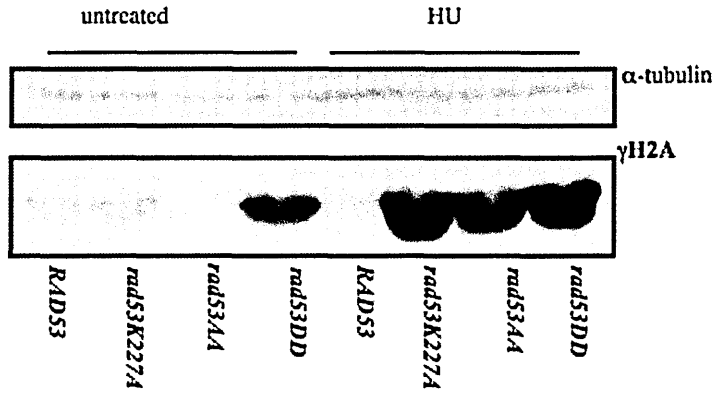


Figure 22. Analisis of S129 phosphorylation of H2A histone in T-loop mutants.

25 μg aliquot of total protein was prepared from cells untreated or treted with 200mM HU and analyzed by western blotting using commercial monoclonal antibodies against S-129 phosphorylated isoform of histone H2A and commercial antibodies against α Tubulin for quantitation and normalization of the intensity of the signals (see Material and Methods).

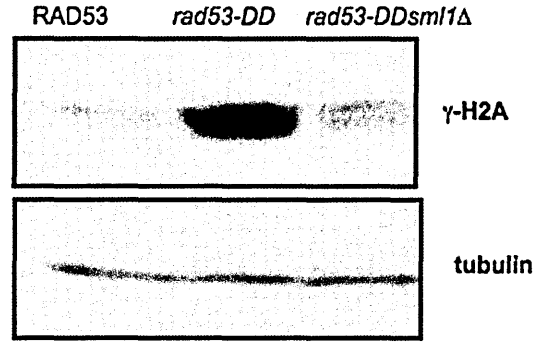


Figure 23. Analysis of H2A phosphorylatiom in DD mutants.

A 25 μg aliquot of total protein was prepared and analyzed by western blotting using commercial monoclonal antibodies against S-129 phosphorylated isoform of histone H2A and commercial antibodies against α Tubulin for quantitation and normalization of the intensity of the signals (see Material and Methods).

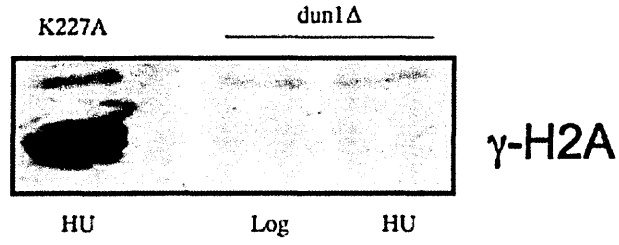


Figure 24. Analysis of H2A phosphorylation in Δdun1 strain.

Cells from dun1Δ strain were treated for 3 hours with 200mM HU. 25μg of total protein were loaded

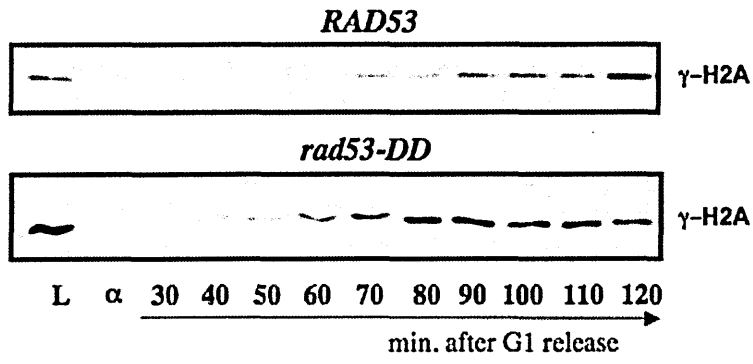


Figure 25. Analysis of phosphorylation of H2A during S phase.

Cells of wild type and *rad53-DD* strains were synchronized in G1 and released in fresh medium at 20°C. A 25μg aliquot of total protein was loaded.

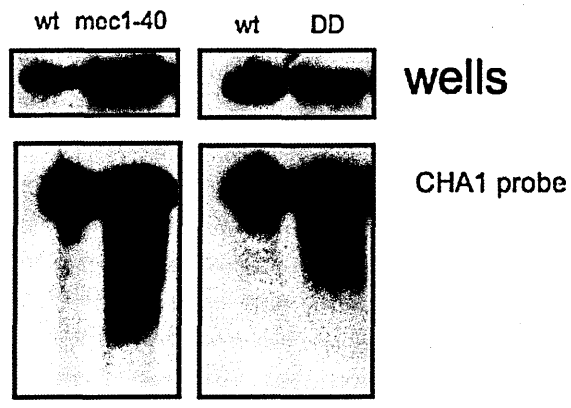


Figure 26. PFGE of wild type, *rad53-DD* and *mec1-40* chromosome III.

Exponentially growing cells of wild type and rad53-DD, and mec1-40 cells at 37°C were fixed in agarose plugs. DNA was extracted and run in a PFGE apparatus. Chromosome III was detected by labeling with CHA1 probe.

Genetic interactions

Given previous observations, we speculated a possible genetic interaction of *rad53-DD* with S-phase checkpoint protein Mrc1 (Mediator of Replication Checkpoint). This protein is a component of replication fork where it is positioned to respond to stalled replication forks and activate the DNA damage checkpoint response since its activity is required to activate Rad53 during replication stress (Alcasabas et al., 2001; Osborn and Elledge, 2003). Its activity is, moreover, required to promote fork progression in untreated conditions (Szyjka et al., 2005).

In budding yeast it is possible to analyze the product of each single meiosis; budding yeast indeed, can exist as an aploid strain (the one that we use routinely to analyze the effect of a mutation) but can exist also as a diploid strain. In certain media (the sporulation plates) diploid cells undergo to sporification and produce four spores delimited in one *ascus*. By isolating each single spore from each single *ascus* it is possible to follow the result of each single meiosis. We crossed a strain containing *rad53-DD* mutation with a strain containing *MRC1* deletion; each mutation is linked to a marker (kanamicine for the *rad53* allele and histidine for *MRC1* deletion).

As shown in figure 27, the combination of *rad53-DD* mutation whit *MRC1* deletion is lethal since no spore carrying both histidine and kanamicine markers were found.

This result suggests that in presence of *rad53-DD* the checkpoint function of *MRC1* is essential. The lethality of the double mutant could be due to the fact that the replication rate in *mrc1*Δ is slow; slow down of replication fork progression could cause a major requirement for the fork-stabilization and protection activity of Rad53 and result in *rad53-DD* background in a even more enhanced phenotype. It would be interesting to test the combination of *mrc1-AQ* mutation whit *rad53-DD*; this allele of *MRC1* has been shown to separate checkpoint functions and replication functions in normal conditions since it shows

a DNA-damage defective phenotype but exhibits a wild type DNA replication rate (Osborn and Elledge, 2003).

We wanted to check the genetic requirement for other checkpoint proteins such as Tel1 and Rad9. These two proteins are known to be involved in the response to double strand breaks. Rad9 is an adaptor protein that transduces the checkpoint signal from Mec1 to Rad53. The interaction between Rad53 and Rad9 is mediated by FHA domains of Rad53. The two FHA recognize PIKK-phosphorylated Rad9 and this interaction is required for catalytic activation of Rad53 and extensive Rad53 autophosphorylation (Durocher et al., 2000a; Sun et al., 1998).

Like Mec1 and ATR, the checkpoint PIKKs Tel1 and ATM, are recruited to free DSB ends through a mechanism dependent on the C terminus of Xrs2. Tel1 and the C terminus of Xrs2 are similarly involved in cell survival and Rad53 phosphorylation after DNA damage. Tel1 association with DNA lesions is required for the activation of DNA damage responses (Nakada et al., 2003).

Neither *RAD9* deletion nor *TEL1* deletion rescued the small colony phenotype or the lethality of *rad53-DD* mutation (figure 27). This result suggests that in absence of Tel1, Mec1 through Rad9 can cause the phosphorylation of *rad53-dd*, while in absence of Rad9 protein, Tel1 is probably doing the job. It would be interesting to analyze the triple mutation.

Since the data produced so far suggested the formation of double strand breaks in *rad53-DD* mutants, we decided to analyze the requirement for Rad52 in this background. Rad52 appears to encode for a protein which has essential functions in homologous sequence-driven recombination; indeed it is required for repair of double strand breaks (Malkova et al., 2005) and its function is required also for gene conversion and meiotic recombination (Jackson and Fink, 1981). Furthermore Rad52 forms foci at DSB or after treatment with ionizing radiations (Lisby et al., 2004); spontaneous foci of Rad52 are also observed in budded cells indicating that Rad52 assembles in response to DNA damage incurred during

DNA replication (Lisby et al., 2003). Deletion of *RAD52* in *rad53-DD* background (figure 28), resulted in a near lethal phenotype suggesting that homologous recombination is essential for the surviving of this strain. This observation is consistent with the fact that *rad53-DD* mutation results in production of DSBs and the recombination is required but not sufficient to maintain chromosomes and to prevent lethal events. These kind of lesions can induce gross chromosomal rearrangement influencing directly genome stability. Furthermore the lethality with *RAD52* is suppressed by deletion of *SML1* (figure 28).

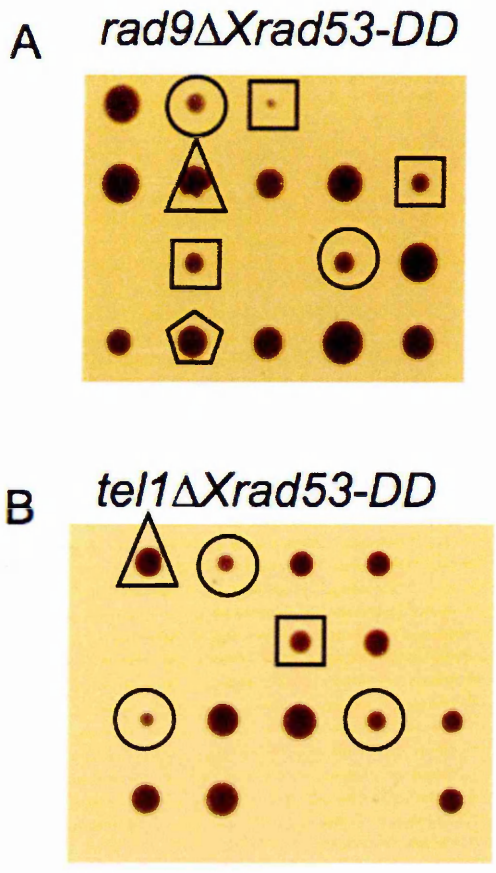


Figure 27. Tetrade analysis of *rad9Δxrad53-DD* and *tel1Δxrad53-DD.*

Aploid cells were mated on YPD plates for 4 hours and zygotes were isolated. Single diploid colonies were than transferred on sporulation plates. After 3 days at 25°C *aschi* were treated with zymoliase to isolate the single spores. After three days of growing at 28°C colonies were analyzed for their genotype. Square: *rad53-DD*, ring: *rad9Δ* (or *tel1Δ)rad53-DD*, triangle:*rad9Δ* or *tel1Δ*.

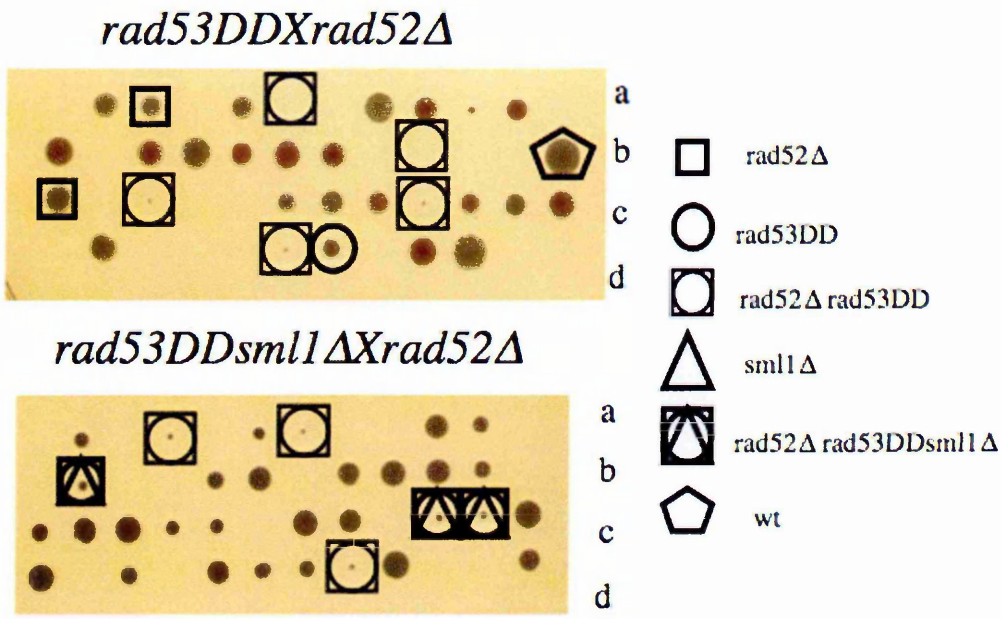


Figure 28. Tetrade analysis of rad53-DDXrad52Δ and rad53-DDXrad52Δsml1Δ.

Aploid cells were mated on YPD plates for 4 hours and zygotes were isolated. Single diploid colonies were than transferred on sporulation plates. After 3 days at 25°C *aschi* were treated with zymoliase to isolate the single spores. After three days of growing at 28°C colonies were analyzed for their genotype

Abundance of RNR-complex inhibitor Sml1

As previously discussed, deletion of *SML1* gene is able to rescue viability of a strain lacking *RAD53*. This protein binds to and inhibits the ribonucleotide reductase complex RNR (Zhao et al., 1998); during replication stress like in presence of hydroxyurea, Rad53, Mec1-dependent phosphorylation of Sml1 causes its degradation to increase deoxynucleoside triphosphate pools. Our previous results led to the idea that the abundance of Sml1 protein in *rad53*-DD cells might represent a limiting factor which in turn causes the accumulation of spontaneous DNA breaks and a de-repression of a checkpoint response even in an unperturbed cell cycle.

It is known that the degradation of Sml1 protein at the G1/S transition is required to drive DNA synthesis, influencing genome replication in unperturbed cell cycle and in response to DNA damage too (Zhao et al., 2001). Significantly, ectopic over-production of Sml1 protein causes a block of DNA synthesis (Zhao et al., 2001).

Although the mechanisms of the process are not known, the degradation of Sml1 is controlled by a Rad53-dependent pathway (Zhao et al., 2001). To test the abundance of Sml1 in *rad53* alleles, we analyzed by western blot a derivative HA-tagged version of Sml1 protein throughout the cell cycle progression.

We found (figure 29) that the kinetic of degradation of Sml1 protein from a transient G1-block induced by alpha factor is affected in *rad53*-DD cells and it likely correlates with a prolonged DNA replication transition, as previously observed in *dun1*Δ and other *rad53* alleles (figure 29 and (Zhao et al., 2001)).

We have also tested the abundance of Sml1 protein in cells blocked in S phase by HU treatment (figure 30). We found that the level of Sml1 protein promptly decreases as cells block DNA synthesis, as previously shown (Zhao et al., 2001), but this is dramatically affected in *rad53*-DD cells where the signal for Sml1 protein is maintained high for hours.

These results support the hypothesis that high level of Sml1 protein in the *rad53*-DD cells affects normal DNA replication during S phase, perhaps due to unbalanced dNTPs pool, leading to the accumulation of spontaneous DNA breaks and to the activation of apical checkpoint kinases, even in an unperturbed cell cycle. However at the moment we cannot exclude that in *rad53-DD* the abundance of Sml1 is due to an enhanced level of gene expression with respect to a defect in promoting its degradation.

It is important to note that double strand breaks formation is not due to the accumulation of Sml1 *per sé* since dun1Δ strains that accumulate sml1 (Zhao et al., 2001) do not show an increase in H2A phosphorylation (figure 24).

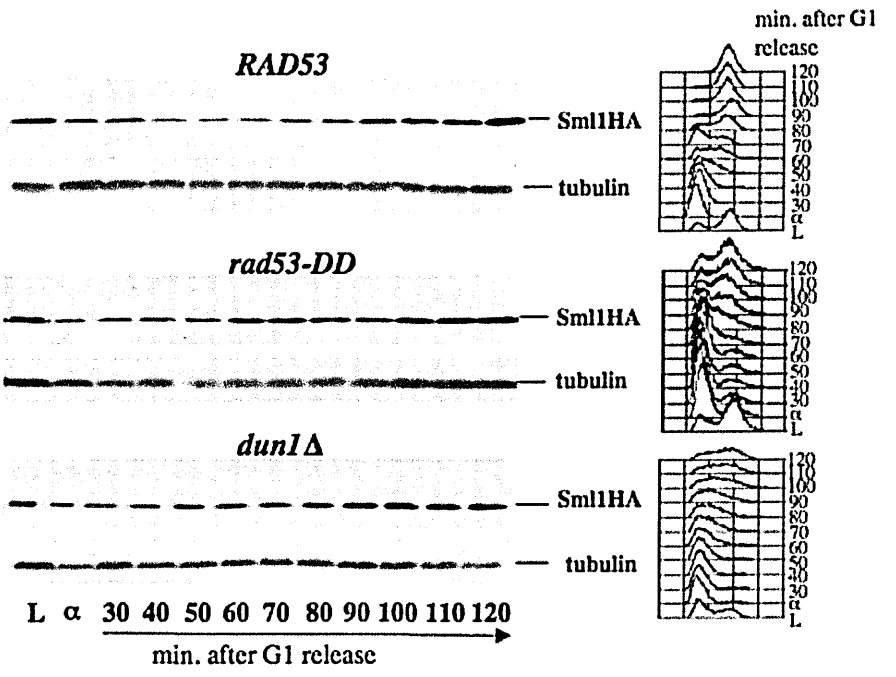


Figure 29. Levels of Sml1 protein in rad53 T-loop phosphosites mutants.

Exponentially growing cultures of the strains CY7075 (wild type), CY7171 (*rad53*-DD) and CY7459 (*dun1*Δ) were pre-synchronized by a-factor treatment and released from the G_1 in YPD. Samples were taken at the time points indicated and analyzed by FACS. A 25 μg aliquot of total protein was prepared and analyzed by western blotting.

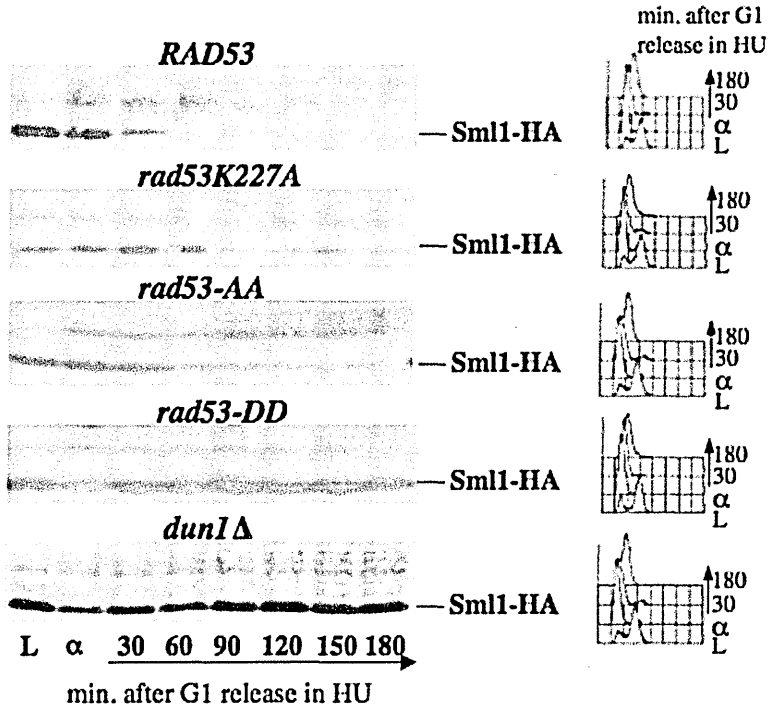


Figure 30. Levels of Sml1 protein in rad53 T-loop phosphosites mutants.

Exponentially growing cultures of the strains CY7075 (wild type), CY7094 (rad53-K227A), CY7096 (rad53-AA), CY7171 (rad53-DD) and CY7459 (dun1Δ) were pre-synchronized by a-factor treatment and released from the G_1 in YPD with 0.2 M HU. Samples were taken at the time points indicated and analyzed by FACS. A 25 mg aliquot of total protein was prepared and analyzed by western blotting.

Physical interaction between Rad53 and Dun1

Dun1 is a checkpoint protein that is mainly involved in the regulation of the transcriptional response following DNA-damage. This response involve induction of elevated transcription of RNR genes (Elledge et al., 1993). Some of the regulation occurs also post-transcriptionnally as discussed before, since Dun1 can phosphorylate Sml1 and induce its proteolysis. Furthermore, Dun1 can interact with Rad53 after MMS treatment and this interaction does not require the kinase activity of Rad53 as demonstrated by Usui and co-workers but is mediated by FHA domain of Dun1 (Bashkirov et al., 2003; Usui and Petrini, 2007).

The effect of over-expression of *rad53-DD* or *rad53-AA* mutants version of *RAD53*, seen in figures 12 and 13 led us to think that the mutation of the T-loop could affect the formation either of homodimers of Rad53 molecules or heterodymers Rad53-Dun1. Thus we wanted to test the possibility that perturbation on T-loop domain of Rad53 could affect the formation of the hetero-complex in hydroxyurea.

I performed a co-immunoprecipitation assay on extracts from wild type cells or *rad53-k227A*, *rad53-AA*, *rad53-DD* in unperturbed condition, in the presence of HU, and after release from HU into fresh medium. In this experiment I immunoprecipitated Dun1-3HA (see experimental procedures) and looked by western blot for the presence of Rad53 protein. In figure 31 we can notice that in the presence of HU wild type and *rad53-DD* proteins are able to interact with Dun1, while the interaction of *rad53-K227A* or *rad53-AA* with Dun1 in these conditions is barely detectable. After release from HU *rad53-DD* mutants are unable to release Dun1. This inability to resolve the Rad53-Dun1 complex could be responsible for the stability of sml1 in HU; Dun1 could be sequestered and maintained blocked. A second explanation is that *rad53-DD* is simply unable to phosphorylate and activate Dun1. Neverthless we propend for the first hypotesis Dun1

protein results at least partially phosphorylated in *rad53-DD* mutants after HU treatment. Dun1 protein has a similar T-loop domain as compaired to Rad53; given this notion we cannot exclude that this phosphorylation is reflecting autokinase activity of the protein that could be stimulated by interaction with Rad53. The defect of *rad53-K227A* and *rad53-AA*, could be due to a defect of Rad53 in activating Dun1 since the level of phosphorylation in these mutants in presence of HU is low, either because of the absence of kinase activity in these mutants or because of a inefficiency in establishing a Rad53-Dun1 complex.

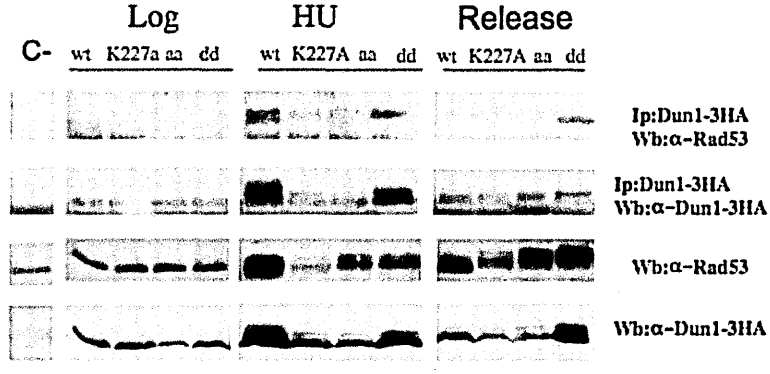


Figure 31. Physical interaction between *rad53-alleles* and Dun1.

Cells of indicated strains were grown in YPD and then treated for three hours with 200mM HU. Cell were then released in fresh medium without drug for one hour. Whole cell extract from indicated strains and a control (C-) strain without tagged version of Dun1 were incubated with beads cross-linked to HA epitope. The immunoprecipitated fractions were then analyzed for the presence of Rad53

Analysis of DNA replication intermediates of rad53-AA and rad53-DD mutants

We decided to analyze the replication intermediates of *rad53-AA* and *rad53-DD* in presence of HU and to compare them with wild type and *rad53-K227A* mutant. In this case I decided to analyze the phenotype of the mutation in *sml1Δ* background in order to avoid asynchrony due to the slow growth phenotype of *rad53-DD* mutant and in order to analyze only the contribution of *rad53-DD* mutation. We took advantage of the bi-dimensional gel technique (see experimental procedure) that allows the separation of the different replication intermediates. When a DNA fragment is being replicated, it assumes different structures that differ from each other by their mass and shape. In particular, when an origin of replication is fired bi-directionally inside the fragment, bubbles shaped structures with increasing mass are formed as the fork proceeds towards the ends. Instead, when a fragment is replicated passively, meaning that a replication fork enters from one extremity, a population of Y-shaped structures with different mass and shape will form. Neutral-neutral two-dimensional agarose gel electrophoresis (2D-gel) technique allows separation and identification of branched DNA molecules according to their mass and shape complexity (Bell and Byers, 1983). This technique further developed by (Brewer and Fangman, 1987) has been used to map origins of DNA replication in yeast chromosomes and to study replication and recombination related DNA structures in many organisms.

We analyzed with this technique replication intermediates of ARS305; *ARS305* is an efficient origin that fires early in S-phase (Newlon et al., 1993; Poloumienko et al., 2001) located on the left harm of chromosome III. Cultures from wild type, or mutant strains were arrested with α-factor and released in fresh medium containing 200mM HU. Samples were taken at the time points indicated in figure 24 and after DNA extraction and digestion

with NcoI (see experimental procedures for details), I performed a first electrophoretic run in order to optimize the separation of DNA molecules by mass, followed by a second run in a perpendicular direction with condition that optimize the separation of DNA molecules by shape. After transfer on a hybridation membrane DNA was visualized by labeling with a probe recognizing a fragment containing ARS305.

Fork reversion has been observed in *E. Coli* at stalled forks in the absence of a topoisomerase or after UV treatment and in *S. cerevisiae rad53* checkpoint mutant after fork stalling in the presence of Hydroxyurea (Courcelle et al., 2003; McGlynn et al., 2001; Sogo et al., 2002).

As shown in figure 32 both mutants showed degeneration of replication intermediates, as we can assess by the disappearance of bubbles, an appearance of homogeneous Y arc and appearance of a cone shape. The same phenotype was already observed in our laboratory in strain lacking kinase activity of Rad53 (Lopes et al., 2001). Indeed, in *rad53-K227A* treated with HU, it is possible to note that the disappearance of bubble molecules coincides with the appearance of a more homogeneus Y arc. This phenomenon is thought to be the result of degradation processes that arise in absence of Rad53 activity. This degradation seems to be due to the inability of *rad53-K227A* to retain at stalled replication forks replisome proteins and seems to depend at least partially on the action of an exonuclease protein, Exo1 (Cotta-Ramusino et al., 2005; Lucca et al., 2004).

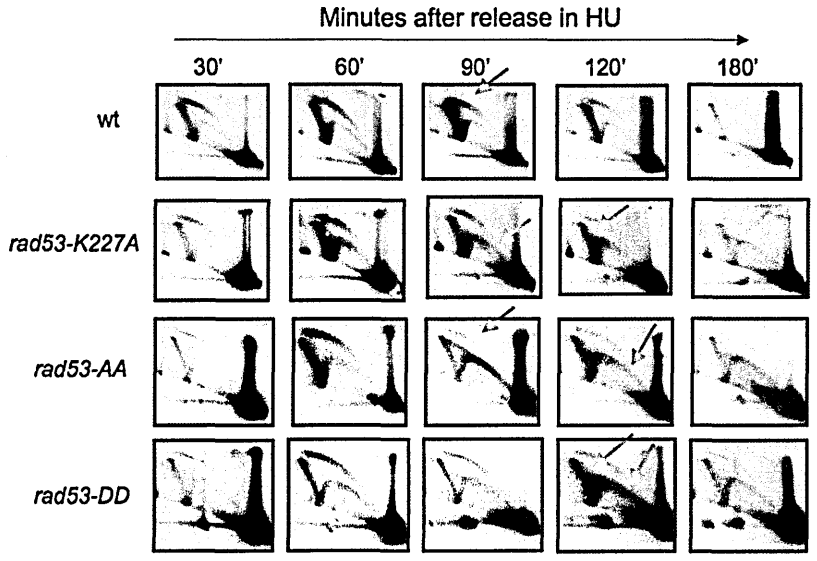


Figure 32. Analysis of replication intermediates in T-loop mutants.

Cells of cy3136 (wt), cy5923 (rad53-AA),, cy5924(rad53-DD) were arrested in G1 and release in fresh medium containing 200mM HU. Samples were taken at the indicated time points and DNA was analyzed by 2D gel technique.

DISCUSSION

New tools to unravel the mechanisms promoting Rad53 activation

Rad53 has been implicated in many aspects of the DNA damage and replication checkpoints (Pellicioli et al., 1999; Sun et al., 1996). Two recent works showed a number of phosphorylation and authophosphorylation sites likely implicated in tuning the kinase activity of the protein (Smolka et al., 2005b; Sweeney et al., 2005). The production of monoclonal antibodies that specifically recognize certain isoforms of Rad53 will be usefull in understanding Rad53 regulation. In this PhD project I have produced a series of monoclonal antibodies that recognize by western blots different forms of the protein. We focused our attention on EL7 anf F9 Mabs. EL7 is able to recognize a wide range of Rad53 isoforms, from dephosphorylated to fully phosphorylated Rad53. The F9 Mab recognizes Rad53 only when the protein is fully active, as it immunoreacts with the wild type protein present in extracts from cells treated with different DNA damaging agents but not from untreated cells. Furthermore the F9 antibody failed to detect the protein in the kinase dead allele *rad53-K227A* even in treated conditions. The bands that appeared in western blotting with F9 always corresponded to active Rad53 as assumed by comparing blotting with ISA (*in situ* kinase assay); this observation strongly suggests that F9 recognize the active form of Rad53. There are two possibilities: either these antibodies recognize the phosphorylated T-loop of the protein or they recognize an epitope of the protein that is exposed when the protein is activated. The F9 failed to recognize the kinase dead alleles *rad53-K227A* and *rad53-DD* and *rad53-AA* because they are unable to perform authophosphorylation. If the second hypotesis its true we should assume that conformational changes induced by activation of the protein are the results of autophosphorylation events instead of in trans phosphorylation mediated since all the kinase dead alleles are still able to be in trans phosphorylated by ATR/ATM related proteins as assumed by the intermediated shift

appreciated on SDS gels. At the moment we cannot distinguish between these two possibilities.

The phosphorylation of Rad53 at different sites is likely to mediate stress specific differences in the extent of kinase activation. Indeed, Chk2 purified after gamma radiation, UV, and HU treatment showed different kinase activities toward Cdc25, one of its best validated substrates (Matsuoka et al., 1998). It will be interesting to analyze in the next future other antibodies trying to identify specific immunoreactive species with extract treated with different drugs or even different concentration of the drugs. Identifying different phosphorylated Rad53 isoforms representing differential states of activation will help to elucidate the role of the kinase in response to different genotoxic stresses. Furthermore it will be very interesting to test the same antibodies in kinetic experiments. For example, after the induction of a single reparable DSB Rad53 starts to be phosphorylated after 2 hours (when the ssDNA is over the treshold of 10 Kb) and reaches its fully activation at 4 hours. When the lesion has been removed the phosphorylated form of Rad53 start disappearing. With the help of these antibodies we should be able to correlate a specific isoform of Rad53 with different activating stages.

EI4 or DO1 Mabs seem to recognize specifically the phosphorylated form of Rad53 that is present in 4NQO treated cells since they don't recognize the phosphorylated band in HU and MMS. We could argue that these particular antibodies specifically recognize the form of Rad53 that is responsible for the response to UV radiations, that is different by the isoform that respond to replication stress. Theoretically we could immunoprecipitate the protein with these antibodies and analyze by mass spectrometry the phosphorylation sites. We could be able to attribute a phosphorylation signature to a specific cellular response involving Rad53. Another possibility will be to use these antibodies to identify, by mass spectrometry, specific partners of specific isoforms of Rad53.

The CI4 clone seems to recognize more than one band in untreated conditions. These Mabs could lead to the demonstration of the basal activity of Rad53 that has been suggested by different observations in literature, but never associated to phosphoryolation of the protein.

Production of new Rad53 alleles

We speculated that affecting one particular phosphorylation site , would affect a specific function of Rad53. We started, mutagenizing by a site-directed strategy, residues in the SQ/TQ motif of Rad53 at the N-terminus of the protein. In particular we identified Threonine 8 and 15 on the basis of their conservation throughout the evolution. Single mutation of these sites did not show any particular phenotype in response to HU and 4NQO treatment. These results suggested a possible redundant function of T8 and T15 and other two trheonine presents in the region T5 and T12. This hypothesis was than demonstrated by a work in which the authors demonstrated that the presence of one out of these four threonine is sufficient to restore viability to wild type levels following HU exposure (Bartek et al., 2001; Lee et al., 2003b). We cannot exclude that the checkpoint defect of these mutants is under the level of detection of our experiments. Observations made in our laboratory (Vanoli and Foiani unpublished results) showed that treatment with such a dose of 4NQO doesn't allow to discriminate Rad53 phosphorylation level in wild type with respect to certain mutants. The use of lower concentration (0,7µg/ml instead of 2µg) amplify the difference in phosphorylation between wild type and mutants further supporting the idea that the extent of Rad53 phosphorylation correlates with the extent of its activation and requirement.

Perturbations of the Kinase domain of Rad53 affect the activity of the protein

RAD53 was described as an essential gene in 1993 by Zheng and co-workers (Zheng et al., 1993). In this study the authors already suggested a role for Rad53 in DNA replication. This suggestion was based on the observation that the phenotype showed by *rad53* alleles was similar to the ones of *cdc* mutants that have defects in the elongation stage of DNA replication.

In 1997 a work by Fay and co-workers showed that low levels of kinase activity (10% relative to wild type protein) are enough to support cell life (Fay et al., 1997). On the other hand in 1993 it was demonstrated that ectopic expression of RAD53 driven by a galactose-inducible promoter is deleterious for cell growth resulting in slow growth rate and an accumulation of cells with multiple buds. All these observations strongly suggest that RAD53 is an essential gene whose activity must by finely controlled since total absence of kinase activity would result in cell death while high level of Rad53 activity inhibit cell growth.

We then analyzed the kinase domain of the protein. The analysis of the primary sequence of the kinase domain revealed that Rad53 is a RD-box kinase containing an activation segment (or T-loop).

For many kinases, activation requires phosphorylation of the activation segment. The kinases that are activated through the activation segment phosphorylation have a conserved arginine preceding the conserved catalytic aspartate in the catalytic loop (Johnson et al., 1996) and for this reason are termed RD kinases. It has been proposed that phosphorylations of residues in the T-loop have the role of introducing negative charges that counteract the positive charge of the arginine and the other amionoacids in the catalytic region contributing thus to the correct conformation of the catalytic and of the substrate-interacting domains. This idea is further supported by two observations: kinases that do not posses the arginine before catalytic aspartic, are not phosphorylated in the T-

loop, and RD-box containing kinases that are not phosphorylated in the T-loop posses negatively charged aminoacids that could predispose the kinase in a constitutive active state.

The activation segment or loop (as defined by its conformation in the secondary structure) is defined as the region between and including two conserved tripeptide motifs (DFG...APE). Interestingly the activation loop showed considerable structural diversity as was shown by the comparison of emerging structural data coming from crystallization experiments; this diversity in both conformation and sequence is probably reflecting the requirement for fine tuning of kinase-specific functions. Most kinases contain specific consensus sites within the activation loop that are important for phosphorylation and dephosphorylation, and are critical in determining the conformation of the loop and, consequently, the activity of the kinase. The activation loop is also a site for protein-protein interactions that can be critical in controlling the localization and regulation of a kinase and its binding partners.

Structural evidences have suggested that the activation segment is able to undergo large conformational changes when the kinase switches between inactive and active states (reviewed in (Hutchins and Clarke, 2004). For example Johnson and co-workers (1996) showed that in the insulin receptor kinase (Irk) the phosphorylation of the activation segment causes the movement of this loop away from the catalytic center and adopt a conformation that allows substrate binding and catalysis that are, in the inactive state, inhibited by the presence of the loop itself.

In the case of CDKs, the fully activation is reached by the interaction with ciclins; this interaction causes a conformational change that causes the moving of the t-loop from the catalytic site and this structure is further stabilized by phosphorylation inside activation segment.

We found in Rad53 sequence the basic motifs of a kinase domain and activation loop APE and DFG as well as RD-box. On the basis of this observation we identified two Threonine

residues (T354-358) that are conserved throughout the evolution that could be the site for auotophosphoylation. Due to the evolutionary conservation of these two residues from yeast to humans, we speculated for them a possible regulative role. The phosphorylation of these two residues could counteract the positive charge of the arginine preceding catalytic aspartic in the RD-box as proposed for human PKA. Starting from these observations we decided to mutagenize by site-directed mutagenesis the two trheonine with aspartate in order to mimic the negative charge that is introduced by the addiction of a phosphate in this position and with alanine, a neutral charged aminoacid that cannot be phosphorylated.

In literature it was demonstrated that mutations inside the kinase domain of Rad53 result in checkpoint defective phenotypes, while only two of them affect growth in normal conditions (*rad53D339A and rad53K227A-D339A*). These two mutants show really low kinase activity demonstrating further that kinase activity of Rad53 is required to support cell growth. Here we isolated two new mutants of Rad53, *rad53-T358D* and *rad53-T354D-T358D* that affect both checkpoint functions and growth rate. All the single and double mutants of Rad53 showed no kinase activity in our experiments. These mutants are likely to retain partial kinase activity with *rad53-AA* allele showing the more kinase activity followed by *rad53-K227A* and *rad53-DD*. This observation is suggested by the analysis of HU sensitivity showed by the mutants and by the fact that *rad53-DD* accumulated dead cells thus demonstrating that the mutant retain the minimal kinases activity required to support cell propagation.

The slow growth phenotype observed in *rad53-DD* and *rad53-T358D* mutants together with the observation that Rad53 showed a slower mobility shift band in untreated conditions led us to the hypothesis that theses mutants could mimic the constitutive active form of Rad53. However a series of observations are against this hypothesis. First of all these mutants are checkpoint defective as detected by HU sensitivity and checkpoint assays in MMS suggesting that the kinase activity of these mutants is affected. Second, the kinase assay showed no detectable incorporation of 32pATP in all the mutants analyzed.

The latter observation cannot be conclusive since, in theory, if the two Threonine are unique the site for phosphorylation we could expect no incorporation in this assay. Two observations are against this hypothesis: the assay was not enough sensitive (rad53-K227A is known to retain some kinase activity); studies by mass spectrometry mapped on Rad53 many other authophosphorylation sites respect the two threonines. In particular it was shown that T354 is site for autophosphorylation together with serine 350 and many others (Sweeney et al., 2005). The authors did not identify T358 that in our hand is the most important. This could be due to the detection level of the technique or to the differences in the treatment of the cells. T358 and T354/T358 could exhibit the same phenotype just because mutation of T358 does not allow conformational changes or modification of T354.

A third observation that rule out the possibility that *rad53-DD* mimics the always active form of Rad53 is that all the phenotypes are recessives. The HU sensitivity, is suppressed by reintroducing wild type Rad53 or even introducing a form of Rad53 that carries two serines instead of the two threonines. These residues can be phosphorylated and the possibility to phosphorylate the T-loop is enough to sustain HU treatment. Furthermore, in diploid cells that carry mutant forms of *RAD53* on one chromosome and the wild type version on the sister, all the phenotypes are rescued (not shown). In fact we didn't observe slow growth as compare to the wild type and even Sml1 degradation was restored to wild type levels further confirming that *rad53-DD* mutation does not confer a "gain of function".

We exploited the possibility that *rad53-T354D* or *rad53-T354D-T358D* could mimic the form of Rad53 that is switched off with the idea that phosphorylation of one or both residues could drive recovery events or the turn off of the checkpoint signal. We excluded this possibility by considering that a strain without Rad53 kinase activity would result in cell death as a strain lacking RAD53.

The analysis of single mutations suggests that perturbation of T358 residue produces a more severe phenotype with respect to substitution of the first threonine. This effect could

be due to the fact that this is the real residue required for regulation of Rad53 or at least this site is the most important in the experimental conditions we tested. Alternatively, modification of this residue causes a conformational change that affects phosphorylation of T354 that has been proposed as a site for autophosphorylation (Sweeney et al 2005). The conformational change induced by the mutation could also affect the exposition of other sites outside the kinase domain thus affecting phosphorylation and/or interaction with specific partners of Rad53.

We then extended the phenotypic analysis to double mutants to have a more clear effect by combining the mutation that had the most severe phenotype in our hands (T358) and the residues that was isolated by mass spectrometry analysis (T354). The double *rad53-AA* mutant showed a defect in checkpoint functions consistent with reduced kinase activity while the double *rad53-DD* mutant was affected either checkpoint function and cell growth in untreated conditions consistently with the idea that the last mutant retains the minimal kinase activity required for cell life.

rad53-DD phenotypes

During the characterization of the newly produced mutants we were able to attribute to *rad53-TD* and *rad53-DD* mutants three different characteristics: these mutants showed phosphorylation of Rad53 protein even in untreated conditions; this phosphorylation is associated with a slow growth phenotype; furthermore we observed H2A phosphorylation in unperturbed conditions.

The phosphorylation of Rad53 in untreated conditions in exponentially growing culture suggested that in these cells there is spurious checkpoint activation due probably to the accumulation of some kind of DNA lesions. We confirmed the dependency of this modification of Rad53 on apical Kinase activities, since the treatment with caffeine

completely abolished modification of Rad53. We than argued that this checkpoint activation could account for cell growth inhibition.

Since Rad53 kinase activiy is required to stabilize replication forks in HU, we speculated a similar function in untreated conditions with specific requirement for Rad53 activity at certain regions of the genome that delay fork progression (such as rDNA). The loss of this vital function of Rad53 would lead to fork collapse similar to what observed in *rad53-K227A* treated with HU leading to the accumulation of ssDNA and gaps that would be transformed in DSB with the transition through S phase. We reasoned that such a production of DSBs could account for checkpoint activation and chromosomal aberrations, deletions and genomic rearrangements that could lead to chromosome loss and cell death. We analyzed the phosphorylation of H2A in all the mutants and we found that *rad53-DD* mutants showed increased H2A phosphorylation in logarithmic growing with respect to wild type and others kinase dead alleles. We further confirmed double strand breaks formation analyzing chromosomes behavior in *rad53-DD* in untreated conditions. We noticed the appearance of DNA species deriving from chromosome III in *rad53-DD,* similarly to what we can observe in a termosensitive allele of MEC1 (mec1-40) shifted at non-permissive temperature. These species are likely due to the accumulation of DSBs at discrete genomic regions that were identified and called replication slow zones.

We then analyzed the cell death rate of these mutants and we found that lethality of these mutants is greatly increased in *rad53-DD* respect to the wild type, further confirming some aberrant processing in these cells.

Ablation of *SML1* rescues *rad53-DD* phenotypes

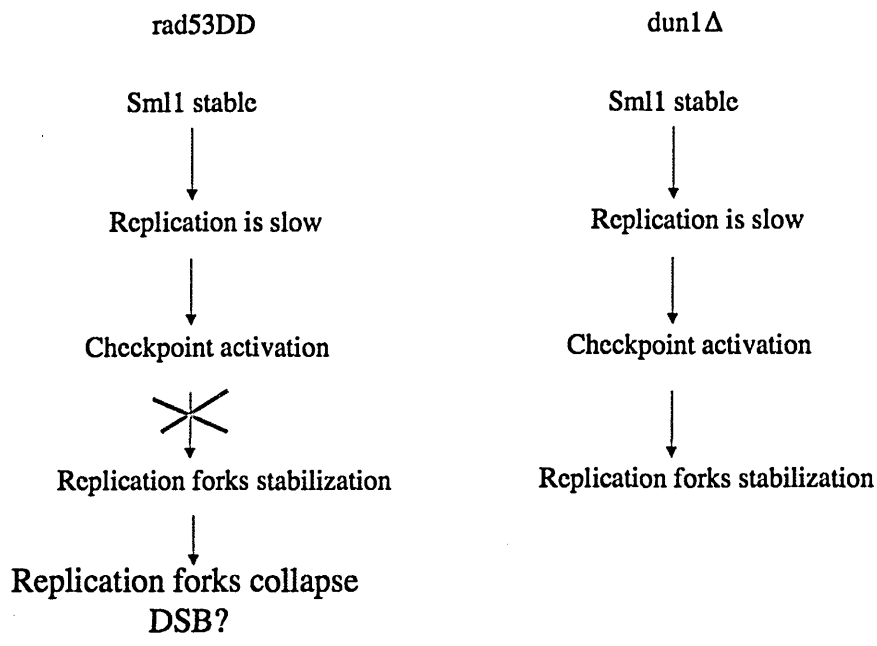
Interestingly, all the phenotypes observed in untreated condition in *rad53-TD* and *rad53-DD* alleles were suppressed by deletion of *SML1*. This protein functions as inhibitor of

ribonucleotide reductase complex (RNR) and its stability is regulated in a Rad53-Dun1 dependent manner. In particular phosphorylation of this protein leads to his ubiquitination and proteasome mediated proteolisis to allow RNR activation and accumulation of dNTPs necessary to sustain DNA replication. In damaging conditions Sml1 is suddenly degraded to allow accumulation of dNTPs required for DNA repair.

By deleting *SML1* we were able to suppress slow growth phenotype, phosphorylation of H2A, modification of Rad53, and cell lethality.

We analyzed the abundance of the protein in untreated and treated conditions in all the mutants and in the control deleted for *DUN1* gene. *rad53-DD* mutant showed aberrant levels of Sml1 protein in untreated and in treated cells with respect to the wild type. The control *dun1*Δ showed the same abundance of Sml1. Nevertheless *dun1*Δ didn't accumulate H2A phosphorylation even if the inhibition of RNR due to the abundance of Sml1 caused a delay in S phase progression as demonstrated by FACS analysis. This phenomenon is probably due to the fact that in these cells Rad53 essential function is preserved. In other word the delay of cell cycle would require the fork stabilization/protection activity of Rad53. In *dun1*Δ, this function is present and forks are probably stabilized and phosphorylation of H2A does not increases, while in *rad53-DD* mutation the replication fork progression is generally affected but the missing Rad53 activity results in the production of double strand breaks (see model below).

All these observations suggest that the essential role of Rad53 relies in its ability to protect replication forks when DNA replication is delayed more than in its function to regulate dNTP pool, as was believed so far.



Model explaining rad53-DD phenotype

Future characterizations

At the moment we miss the formal demonstration that the cause of checkpoint activation in *rad53-DD* in unperturbed conditions is due to DSBs formation due to unstable replication forks.

The fact that *rad53-DD* mutation combined with the absence of Rad52, required for all kind of homologous recombination, strongly support the hypothesis of DSBs formations. Nevertheless I was not able to detect any replication fork collapse at rDNA, even if preliminary results suggest an increase of recombination in this genomic location. This

difficulty could be due to the asynchrony of the cell or to the fact that replication fork collapse happens stochastically and randomly in the genome.

For *mec1-40* Cha and co-workers (2002) were able to show that DSBs are formed at particular regions of the genome that physiologically slow down replication fork progression, the so-called replication slow zones (RSZ). The fork stalling in unchallenged *mec1-40* cells does not involve immediate replication fork collapse and this correlates with the fact that these cells does not show immediately cell death after the passage through S phase at non permissive temperature. We could speculate the same effect for *rad53-DD* mutation.

The combination of *rad53-DD* mutation with *MRC1* deletion resulted in cell death. It has been demonstrated that Mrc1 function is essential to perform S-phase checkpoint. This result suggests that *rad53-DD* mutation causes a requirement for intra-S checkpoint functions suggesting that phosphorylation of Rad53 in untreated conditions could be due to DNA damage-dependent checkpoint (G2/M) and/or to intra S checkpoint. We propend for the first hypothesis since the cells accumulated in untreated conditions as large budded consistently with a G2/M block caused by DSBs formation.

Preliminary results suggest that deletion of *PIF1* in *rad53-DD* background restore growing rate similar to that of the wild type. Pif1 is a DNA helicase that is involved in the maintenance of mitochondrial DNA (Cheng et al., 2007), and is also involved in inhibiting *de novo* telomere addiction to a DSB and telomerase-mediated telomere addiction (Boule et al., 2005; Schulz and Zakian, 1994). Furthermore Pif1 inhibits the fork movement through the replication fork barrier (RFB) (Ivessa et al., 2003). We could explain this suppression in different ways. Firstly the absence of *PIF1* could favor *de novo* telomere addiction to the formed double strand breaks; it would be interesting to analyze phosphorylation of H2A in the double mutants as well as the HU sensitivity. Alternatively the absence of *PIF1* could allow faster replication forks progression through RFB diminishing thus the requirement for Rad53 activity in stabilizing replication forks.

One other possibility is that DNA damage arises from mitochondrial DNA damage. It would be interesting to analyze these mutants for mitochondria maintenance since *rad53* mutants show high rates of mitochondria loss (Zhao et al., 2001).

An intriguingly possibility is that checkpoint activation in *rad53-TD* and *rad53-DD* mutants could reflect apoptotic events due to the accumulation of DNA damage in these cells. In yeast a caspase (Yca1) has been described as responsible for some apoptotic phenotypes. Indeed hydrogen peroxide treatment induces apoptosys and a caspese-like activity that is abrogated in *YCA1* deleted strains (Madeo et al., 2002).

To better understand the nature and the source of the DNA damage accumulating in r*ad53-DD* mutants we will perform a synthetic genetic array (SGA) screening trying to identify suppressor of some or all phenotypes associates with this mutation.

It will be interesting to analyze the role of NLS in Rad53 regulation. Deletion of C-terminal portion of the protein doesn't cause cell lethality suggesting that the essential function of Rad53 is in the cytoplasm (Fay et al., 1997). Nevertheless the authors did not demonstrate that nuclear import was really abolished in these mutants. A putative site for phosphorylation was identified in this region with a possible role for Rad53 regulation (Smolka et al., 2005a). Furthermore Chk2 has been shown to have a phosphorylation site that drives its degradation (Kass et al., 2007). It would be interesting to analyze Rad53 sequence for the presence of the same site.

Concluding remarks

Rad53 is the main transducer of DNA damage mediated checkpoint signal in budding yeast. In higher eukaryotes such as in humans more than one protein have evolved to transduce the signal from different kind of DNA lesions. In mammals the ATM/Chk2 pathway is mainly activated after DNA double strand breaks, whereas the ATR/Chk1

pathway responds to DNA replication stress. Hydroxyurea, aphidicolin and UV are commonly used to induce ATR activation, which, in turn phosphorylate its downstream target Chk1. However, crosstalk exists between those two pathways, and most DNA damaging agents activate both.

In *Schizosaccharomyces pombe* Cds1 is the hortologue of Rad53 and Chk2 and seems to be the major effector responsible for most of the biological effects of the replication checkpoint. Chk1 protein is instead responsible for G2/M checkpoint but not for S-phase checkpoint (Willson et al., 1997).

Rad53 has evolved as the unique central player in budding yeast that can integrate signals from different kinases responding to different kind of DNA lesions. This protein has the unique characteristic of containing two FHA domains further demonstrating the ability of this kinase to act in different pathways. As already discussed above, kinase activity of Rad53 must be finely and timely tuned. The presence of residues in its T-loop, that are likely target for phosphorylation, suggests one mechanism to regulate its activity.

Furthermore this protein seems to be finely tuned in its activation and deactivation. Mutants of this protein are particularly interesting to analyze because they could by useful in separating different function of Rad53 and elucidating the mechanisms that regulate its activation and inactivation. Some evidences have been collected on the mechanism of activation of the protein but the processes regulating its activation are not really understood.

Some protein phosphatases have been involved in its deactivation Ptc2, Ptc3, but they don't seem to be the only regulators of Rad53 activity (Guillemain et al., 2007).

Another protein influencing Rad53 activity is Pph3 even if the mechanisms of such regulation are still not clear (Keogh et al., 2006; O'Neill et al., 2007). The analysis of the mutants could be helpful in blocking some transient interactions or even to identify the precise site of interaction or precise sites of modification required for interaction or release of complexes.

Both for hChk2 and spCds1 it has been proposed a two steps mechanism of activation. In a first step Cds1 or Chk2 interact with the adaptor protein (Mrc1 or Brc2). This interaction leads to phosphorylation of Rad53-hortologues and promotes oligomerization that triggers autophosphorylation events that result in the release of fully active molecules (Xu et al., 2002; Xu et al., 2006).

In the case of Rad53 activation it has been demonstrated that Rad9 and Mrc1 are required to promote the activation of the protein (Alcasabas et al., 2001; Sun et al., 1998) but a similar mechanism of oligomerization has not yet been demonstrated.

The characterization of the T-loop allowed us to gain new insights into the essential role of Rad53 kinase. Our data, suggest that the primary role of Rad53 is to regulate dNTP pool and to stabilize replication forks even in untreated conditions.

APPENDIX

RESULTS

Ypa1: a new player in Rad53 regulation?

Work in our laboratory has previously demonstrated the requirement for CDK1 activity in the maintenance of checkpoint activation following a DSB (Ira et al., 2004).

Ypa1 protein was proposed as a possible target for CDK1 regulation from a genome wide study that took advantage of a CDK mutant sensitive to an ATP analog (Ubersax et al., 2003). Ypa1 (yeast phosphotyrosil phosphatase activator) is a prolyl-cis-trans isomerase and has been reported to activate PP2A phosphatase (Fellner et al., 2003). Furthermore Ypa1 was shown to interact with Pph3.

I firstly speculated that YPA1 could regulate *in vivo* the activity of the phosphatase PP2A-like, Pph3. Pph3 was proposed to dephosphorylate H2A after a single DSB and in absence of pph3 the inactivation of both H2A and Rad53 are affected (Keogh et al., 2006). I analyzed the behavior of *ypa1*Δ in the same background and in the same experimental conditions. In particular the genetic background allows the introduction of a single DSB at a specific genomic location by the over-expression of HO endonuclease mediated by GAL1 promoter. The double strand break can be repaired through homologous recombination with a cassette placed on the same chromosome. Cells were grown in YP-lactate to repress the GAL1 promoter and DSB formation was induced by the addiction of 2% galactose. Samples were taken every 2 hours until 10 hours after the introduction of the DSB; an addictional time point was taken at 24 hours to address a possible defect in adaptation. In figure 33 it is possible to note that wild type cells activate checkpoint response and arrest cell cycle in G2/M phase. At 8 hours wild type starts to

dephosphorylate Rad53 concomitantly with cells re-entering cell cycle. In *ypa1*Δ cells the cell cycle arrest was more pronounced consistently with a more extensive Rad53 phosphorylation. At 24 hours Rad53 was completely dephosphorylated and cells showed a logarithmic distribution in the cell cycle. The defect observed in *ypa1*Δ strain was exactly the same observed in pph3Δ in (Keogh et al., 2006) supporting thus our hypothesis that Ypa1could regulate Pph3. To test the hypothesis that Ypa1 was regulated in a CDK1-dependent manner I performed a different experiment. In Ira et al 2004 it has been shown that deactivation of CDK1 in presence of an irrepairable DSB results in Rad53 deactivation underlying the requirement for CDK1 activity to maintain checkpoint signal; I repeated the same experiment in a ypa1Δ background. In particular cells carrying *cdc28-as* allele (sensitive to the treatment with 1-NMPP1) or the double mutant *cdc28*-as *ypa1*Δ were arrested in G2/M phase by the introduction of an irreparable DSB. After checkpoint activation, detected by the fully activation of Rad53 (figure 34), I divided the cultures in two and I added 1-NMMP1 or DMSO as a control. The result is shown in figure 34; Rad53 phosphorylation seems to be less affected by inhibition of CDK1 activity in ypa1Δ background with respect to the wild type. This effect is mild but reproducible since it was observed in independent experiments and with different independent clones. In yeast, Ypa2 seems to have a redundant function to Ypa1 and the presence of this protein could account for the mild effect observed. Unfortunately the double mutant is lethal and the analysis of the complete absence of *YPA* function will require the employment of conditional mutations.

In budding yeast, the replication checkpoint response requires the Mec1-dependent activation of Rad53 protein kinase (Foiani et al., 2000; Lowndes and Murguia, 2000; Pellicioli et al., 1999). Active Rad53 slows down DNA synthesis and prevents firing of late origins of replication (Santocanale and Diffley, 1998). Furthermore, Rad53 is needed to maintain stable replication fork-replisome association (Lucca et al., 2004), to prevent the accumulation of aberrant intermediates (Lopes et al., 2001; Sogo et al., 2002) and to de-

repress the transcription of damage inducile genes (Aboussekhra et al., 1996; Huang et al., 1998). Presumably the loss of these functions is the cause of the lethality in *rad53-K227A* mutants exposed to HU.

In our laboratory Rachel Jossen performed a screening to identify genome wide KO mutation rescuing the HU sensitivity of *rad53-K227A* in order to reveal genetic pathways regulating or regulated by Rad53. She was able to identify several classes of suppressor on the basis of their ability to rescue HU sensitivity and among them she identified also *YPA1*. One of the classes she identified contains genes that affect Rad53 phosphorylation status. Among them she was able to identify Pph3/Psy2 phosphatases. We developed an adaptation assay to test the phosphorylation status of Rad53 (shown in figure 35); cells were arrested in α-factor and released in fresh medium containing 50mM HU with or without nocodazole and in a medium without any drug. At different time points samples were taken for FACS analysis and protein extracts. We analyzed the phosphorylation status of Rad53 as assessed by western blotting analysis with EL7 Mabs, and the cell cycle progression. As shown in figure 35, we noticed that wild type is able to adapt to presence of HU since it is able to re-enter cell cycle and switch off Rad53. The cells are still able to respond to presence of DNA stressing agents as when they reach the new S-phase reactivate Rad53. In presence of nocodazole, a drug that inhibit microtubule polymerization and arrest cell cycle in G2, wild type cells do not phosphorylate Rad53 anymore.

Since Ypa1 is reported to work with Cla4 in regulating G2/M transition in the so-called morphogenetic checkpoint, we speculated for Ypa1 a role in this pathway. I will perform some experiments to address this issue. In particular I want to test if HU sensitivity showed by this strain is affected by deletion of Swe1.

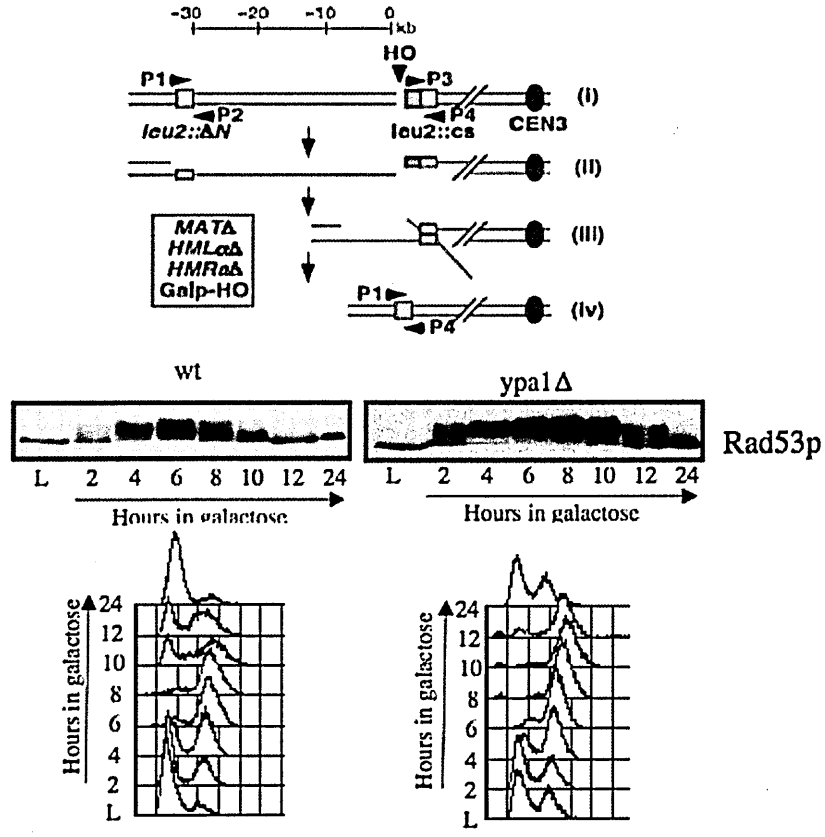


Figure 33. YPA1 is required for recovery after a DSB.

Cells were grown in YP-lactate medium and galactose 2% was added to induce DSB. Checkpoint activation was monitored by Rad53 phosphorylation and cell cycle profile.

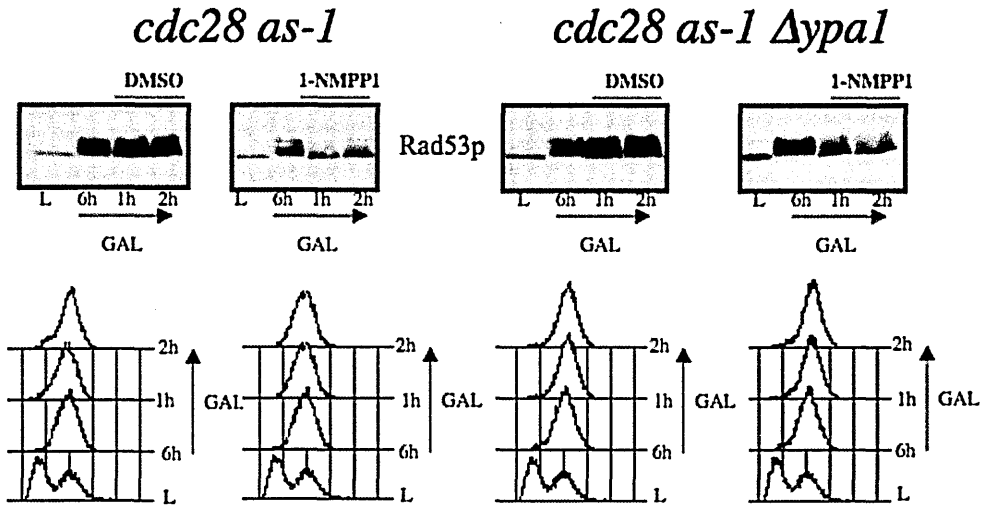


Figure 34. Requirement of Ypa1 in CDK1-mdiated checkpoint maintenance.

Cells of cdc28 as-1 and cdc28 as-1ypa1Δ were grown in YP-lactate. 2% galactose was added to the cultures and after 6 hours the cultures were split in two. 1-NMPP1 was added or DMSO as a control. Rad53 phosphorylation was monitored by western blot and cell cycle phase was checked by FACS.

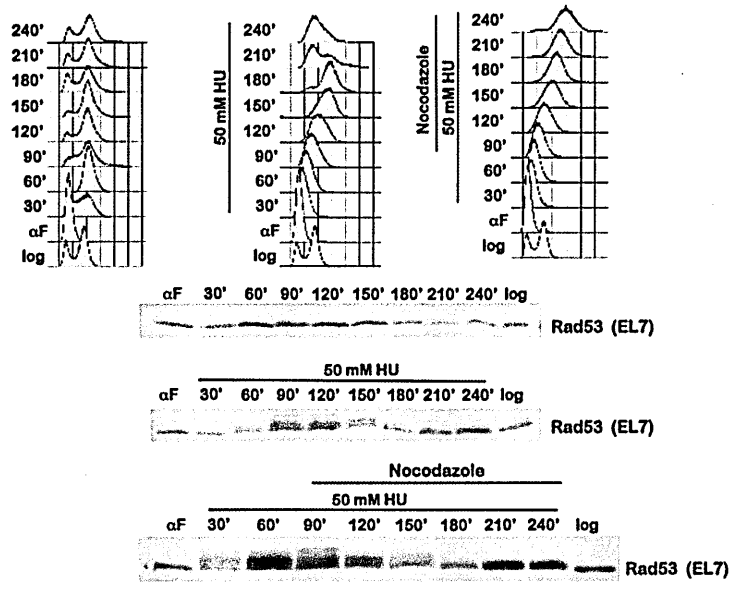


Figure 35. Experimental system to score Rad53 phosphorylation during adaptation to HU.

Cells were arrested in G1 and released in medium containing 50mM HU, without or with nocodazole. FACS analysis and western blot to detect Rad53 phosphorylation status are shown.

DISCUSSION

We were able to uncover a role for YPA1 in the regulation of Rad53 that had not previously described. This gene encode for a prolyl-cic-trans-isomerase that is able to activate *in vitro* PP2A (Van Hoof et al., 2005). As a first hypothesis we though that Ypa1 could drive dephosphorylation of H2A end or Rad53 through the action of Pph3. This protein is a PP2A-like phosphatase that has been suggested to cause H2A dephosphorylation and interact *in vitro* with Ypa1. Pph3 has also been involved in Rad53 dephosphorylation as suggested by persistent phosphorylation of Rad53 after release from MMS in pph3Δ mutants.

Preliminary recovery experiments on *ypa1*Δ after MMS treatment, showed a partial defect in the dephosphorylation of Rad53; this defect was much more evident and persistent in pph3Δ cells suggesting either that Ypa1 does not function to regulate Rad53 phosphorylation status through Pph3 or that Pph3 is not the unique target regulated in a Ypa1-dependent way.

Another intriguingly possibility is that Ypa1 regulate rad53 phosphorylation in a morphogenic checkpoint-mediated pathway. This possibility is supported by the observation that Ypa1 is lethal in combination with Cla4 and that this lethality is suppressed by deletion of Swe1.

These observations place Ypa1 upstream of CDK1 in a signal transduction cascade even if we cannot exclude for Ypa1 the same mechanisms that is regulating Swe1 in the sense that Swe1 phosphorylates Cdc28 but is also a target for CDK1 mediated phosphorylation. Further investigations are required to understand the real role of Ypa1 in checkpoint activation and maintenance. In particular it remains to be understood if deletion of *YPA1* gene influences the efficiency of DSB repair.

All these data are useful to construct the complicated picture of the mechanisms, proteins, processes that are involved in modulating Rad53 activity.

BIBLIOGRAPHY

Aboussekhra, A., Vialard, J. E., Morrison, D. E., de la Torre-Ruiz, M. A., Cernakova, L., Fabre, F., and Lowndes, N. F., 1996, A novel role for the budding yeast RAD9 checkpoint gene in DNA damage-dependent transcription, *Embo J* **15**(15):3912-22.

Abraham, R. T., 2001, Cell cycle checkpoint signaling through the ATM and ATR kinases, *Genes Dev* **15**(17):2177-96.

Agarwal, R., Tang, Z., Yu, H., and Cohen-Fix, O., 2003, Two distinct pathways for inhibiting pds1 ubiquitination in response to DNA damage, *J Biol Chem* **278**(45):45027-33.

Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bousset, K., Furuya, K., Diffley, J. F., Carr, A. M., and Elledge, S. J., 2001, Mrc1 transduces signals of DNA replication stress to activate Rad53, *Nat Cell Biol* **3**(11):958-65.

Amon, A., 1999, The spindle checkpoint, *Curr Opin Genet Dev* **9**(1):69-75.

Aylon, Y., Liefshitz, B., and Kupiec, M., 2004, The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle, *Embo J* **23**(24):4868-75.

Bailis, J. M., and Roeder, G. S., 2000, Pachytene exit controlled by reversal of Mek1-dependent phosphorylation, *Cell* **101**(2):211-21.

Bartek, J., Falck, J., and Lukas, J., 2001, CHK2 kinase--a busy messenger, *Nat Rev Mol Cell Biol* **2**(12):877-86.

Bashkirov, V. I., Bashkirova, E. V., Haghnazari, E., and Heyer, W. D., 2003, Direct kinase-to-kinase signaling mediated by the FHA phosphoprotein recognition domain of the Dun1 DNA damage checkpoint kinase, *Mol Cell Biol* **23**(4):1441-52.

Bell, L., and Byers, B., 1983, Separation of branched from linear DNA by two-dimensional gel electrophoresis, *Anal Biochem* **130**(2):527-35.

Bird, A. W., Yu, D. Y., Pray-Grant, M. G., Qiu, Q., Harmon, K. E., Megee, P. C., Grant, P. A., Smith, M. M., and Christman, M. F., 2002, Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair, *Nature* **419**(6905):411-5.

Boule, J. B., Vega, L. R., and Zakian, V. A., 2005, The yeast Pif1p helicase removes telomerase from telomeric DNA, *Nature* **438**(7064):57-61.

Brewer, B. J., and Fangman, W. L., 1987, The localization of replication origins on ARS plasmids in S. cerevisiae, *Cell* **51**(3):463-71.

Carr, A. M., 2002, DNA structure dependent checkpoints as regulators of DNA repair, *DNA Repair (Amst)* **1**(12):983-94.

Celeste, A., Difilippantonio, S., Difilippantonio, M. J., Fernandez-Capetillo, O., Pilch, D. R., Sedelnikova, O. A., Eckhaus, M., Ried, T., Bonner, W. M., and Nussenzweig, A., 2003, H2AX haploinsufficiency modifies genomic stability and tumor susceptibility, *Cell* **114**(3):371-83.

Cha, R. S., and Kleckner, N., 2002, ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones, *Science* **297**(5581):602-6.

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., and Thelander, L., 2003, Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase, *Cell* **112**(3):391-401.

Chai, B., Huang, J., Cairns, B. R., and Laurent, B. C., 2005, Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair, *Genes Dev* **19**(14):1656-61.

Cheng, X., Dunaway, S., and Ivessa, A. S., 2007, The role of Pif1p, a DNA helicase in Saccharomyces cerevisiae, in maintaining mitochondrial DNA, *Mitochondrion* **7**(3):211-22.

Cheung, W. L., Turner, F. B., Krishnamoorthy, T., Wolner, B., Ahn, S. H., Foley, M., Dorsey, J. A., Peterson, C. L., Berger, S. L., and Allis, C. D., 2005, Phosphorylation of histone H4 serine 1 during DNA damage requires casein kinase II in S. cerevisiae, *Curr Biol* **15**(7):656-60.

Chowdhury, D., Keogh, M. C., Ishii, H., Peterson, C. L., Buratowski, S., and Lieberman, J., 2005, gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair, *Mol Cell* **20**(5):801-9.

Clarke, D. J., Segal, M., Andrews, C. A., Rudyak, S. G., Jensen, S., Smith, K., and Reed, S. I., 2003, S-phase checkpoint controls mitosis via an APC-independent Cdc20p function, *Nat Cell Biol* **5**(10):928-35.

Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P., 2005, The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends, *J Biol Chem* **280**(46):38631-8.

Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P., 2006, The Saccharomyces cerevisiae Sae2 protein negatively regulates DNA damage checkpoint signalling, *EMBO Rep* **7**(2):212-8.

Cohen-Fix, O., and Koshland, D., 1997, The anaphase inhibitor of Saccharomyces cerevisiae Pds1p is a target of the DNA damage checkpoint pathway, *Proc Natl Acad Sci U S A* **94**(26):14361-6.

Collura, A., Blaisonneau, J., Baldacci, G., and Francesconi, S., 2005, The fission yeast crb2/chk1 pathway coordinates the DNA damage and spindle checkpoint in response to replication stress induced by topoisomerase I inhibitor, *Mol Cell Biol* **25**(17):7889-99.

Cortez, D., 2003, Caffeine inhibits checkpoint responses without inhibiting the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases, *J Biol Chem* **278**(39):37139-45.

Cotta-Ramusino, C., Fachinetti, D., Lucca, C., Doksani, Y., Lopes, M., Sogo, J., and Foiani, M., 2005, Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells, *Mol Cell* **17**(1):153-9.

Courcelle, J., Donaldson, J. R., Chow, K. H., and Courcelle, C. T., 2003, DNA damage-induced replication fork regression and processing in Escherichia coli, *Science* **299**(5609):1064-7.

de Bettignies, G., and Johnston, L. H., 2003, The mitotic exit network, *Curr Biol* **13**(8):R301.

den Elzen, N., Kosoy, A., Christopoulos, H., and O'Connell, M. J., 2004, Resisting arrest: recovery from checkpoint arrest through dephosphorylation of Chk1 by PP1, *Cell Cycle* **3**(5):529-33.

den Elzen, N. R., and O'Connell, M. J., 2004, Recovery from DNA damage checkpoint arrest by PP1-mediated inhibition of Chk1, *Embo J* **23**(4):908-18.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., and Elledge, S. J., 1998, Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway, *Genes Dev* **12**(18):2956-70.

Downs, J. A., Allard, S., Jobin-Robitaille, O., Javaheri, A., Auger, A., Bouchard, N., Kron, S. J., Jackson, S. P., and Cote, J., 2004, Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites, *Mol Cell* **16**(6):979-90.

Downs, J. A., Lowndes, N. F., and Jackson, S. P., 2000, A role for Saccharomyces cerevisiae histone H2A in DNA repair, *Nature* **408**(6815):1001-4.

Du, L. L., Moser, B. A., and Russell, P., 2004, Homo-oligomerization is the essential function of the tandem BRCT domains in the checkpoint protein Crb2, *J Biol Chem* **279**(37):38409-14.

Duncker, B. P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S. M., 2002, An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing, *Proc Natl Acad Sci U S A* **99**(25):16087-92.

Durocher, D., Smerdon, S. J., Yaffe, M. B., and Jackson, S. P., 2000a, The FHA domain in DNA repair and checkpoint signaling, *Cold Spring Harb Symp Quant Biol* **65**:423-31.

Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J., and Yaffe, M. B., 2000b, The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms, *Mol Cell* **6**(5):1169-82.

Elledge, S. J., 1996, Cell cycle checkpoints: preventing an identity crisis, *Science* **274**(5293):1664-72.

Elledge, S. J., Zhou, Z., Allen, J. B., and Navas, T. A., 1993, DNA damage and cell cycle regulation of ribonucleotide reductase, *Bioessays* **15**(5):333-9.

Fay, D. S., Sun, Z., and Stern, D. F., 1997, Mutations in SPK1/RAD53 that specifically abolish checkpoint but not growth-related functions, *Curr Genet* **31**(2):97-105.

Fellner, T., Lackner, D. H., Hombauer, H., Piribauer, P., Mudrak, I., Zaragoza, K., Juno, C., and Ogris, E., 2003, A novel and essential mechanism determining specificity and activity of protein phosphatase 2A (PP2A) in vivo, *Genes Dev* **17**(17):2138-50.

Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M., 2002, Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae, *Nat Biotechnol* **20**(3):301-5.

Foiani, M., Pellicioli, A., Lopes, M., Lucca, C., Ferrari, M., Liberi, G., Muzi Falconi, M., and Plevani, P., 2000, DNA damage checkpoints and DNA replication controls in Saccharomyces cerevisiae, *Mutat Res* **451**(1-2):187-96.

Galgoczy, D. J., and Toczyski, D. P., 2001, Checkpoint adaptation precedes spontaneous and damage-induced genomic instability in yeast, *Mol Cell Biol* **21**(5):1710-8.

Garvik, B., Carson, M., and Hartwell, L., 1995, Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint, *Mol Cell Biol* **15**(11):6128-38.

Gietz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A., 1995, Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure, *Yeast* **11**(4):355-60.

Gilbert, C. S., Green, C. M., and Lowndes, N. F., 2001, Budding yeast Rad9 is an ATP-dependent Rad53 activating machine, *Mol Cell* **8**(1):129-36.

Gingras, A. C., Caballero, M., Zarske, M., Sanchez, A., Hazbun, T. R., Fields, S., Sonenberg, N., Hafen, E., Raught, B., and Aebersold, R., 2005, A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity, *Mol Cell Proteomics* **4**(11):1725-40.

Guillemain, G., Ma, E., Mauger, S., Miron, S., Thai, R., Guerois, R., Ochsenbein, F., and Marsolier-Kergoat, M. C., 2007, Mechanisms of checkpoint kinase Rad53 inactivation after a double-strand break in Saccharomyces cerevisiae, *Mol Cell Biol* **27**(9):3378-89.

Harrison, J. C., and Haber, J. E., 2006, Surviving the breakup: the DNA damage checkpoint, *Annu Rev Genet* **40**:209-35.

Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J., 1989, A simple method for site-directed mutagenesis using the polymerase chain reaction, *Nucleic Acids Res* **17**(16):6545-51.

Hochwagen, A., Tham, W. H., Brar, G. A., and Amon, A., 2005, The FK506 binding protein Fpr3 counteracts protein phosphatase 1 to maintain meiotic recombination checkpoint activity, *Cell* **122**(6):861-73.

Huang, M., Zhou, Z., and Elledge, S. J., 1998, The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor, *Cell* **94**(5):595-605.

Hutchins, J. R., and Clarke, P. R., 2004, Many fingers on the mitotic trigger: post-translational regulation of the Cdc25C phosphatase, *Cell Cycle* **3**(1):41-5.

Huyen, Y., Zgheib, O., Ditullio, R. A., Jr., Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Sheston, E. A., Mellert, H. S., Stavridi, E. S., and Halazonetis, T. D., 2004, Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks, *Nature* **432**(7015):406-11.

Ira, G., Pellicioli, A., Balijja, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N. M., Haber, J. E., and Foiani, M., 2004, DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1, *Nature* **431**(7011):1011-7.

Ivanov, E. L., Sugawara, N., White, C. I., Fabre, F., and Haber, J. E., 1994, Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae, *Mol Cell Biol* **14**(5):3414-25.

Ivessa, A. S., Lenzmeier, B. A., Bessler, J. B., Goudsouzian, L. K., Schnakenberg, S. L., and Zakian, V. A., 2003, The Saccharomyces cerevisiae helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes, *Mol Cell* **12**(6):1525-36.

Jackson, J. A., and Fink, G. R., 1981, Gene conversion between duplicated genetic elements in yeast, *Nature* **292**(5821):306-11.

Jiricny, J., 1998, Eukaryotic mismatch repair: an update, *Mutat Res* **409**(3):107-21.

Johnson, L. N., Noble, M. E., and Owen, D. J., 1996, Active and inactive protein kinases: structural basis for regulation, *Cell* **85**(2):149-58.

Kass, E. M., Ahn, J., Tanaka, T., Freed-Pastor, W. A., Keezer, S., and Prives, C., 2007, Stability of checkpoint kinase 2 is regulated via phosphorylation at serine 456, *J Biol Chem* **282**(41):30311-21.

Kaye, J. A., Melo, J. A., Cheung, S. K., Vaze, M. B., Haber, J. E., and Toczyski, D. P., 2004, DNA breaks promote genomic instability by impeding proper chromosome segregation, *Curr Biol* **14**(23):2096-106.

Keogh, M. C., Kim, J. A., Downey, M., Fillingham, J., Chowdhury, D., Harrison, J. C., Onishi, M., Datta, N., Galicia, S., Emili, A., Lieberman, J., Shen, X., Buratowski, S., Haber, J. E., Durocher, D., Greenblatt, J. F., and Krogan, N. J., 2006, A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery, *Nature* **439**(7075):497-501.

Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Johnston, L. H., and Sugino, A., 2000, Characterization of the yeast Cdc7p/Dbf4p complex purified from insect cells. Its protein kinase activity is regulated by Rad53p, *J Biol Chem* **275**(45):35051-62.

Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B., 1999, Substrate specificities and identification of putative substrates of ATM kinase family members, *J Biol Chem* **274**(53):37538-43.

Kondo, T., Wakayama, T., Naiki, T., Matsumoto, K., and Sugimoto, K., 2001, Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms, *Science* **294**(5543):867-70.

Kornbluth, S., Smythe, C., and Newport, J. W., 1992, In vitro cell cycle arrest induced by using artificial DNA templates, *Mol Cell Biol* **12**(7):3216-23.

Krejci, L., Van Komen, S., Li, Y., Villemain, J., Reddy, M. S., Klein, H., Ellenberger, T., and Sung, P., 2003, DNA helicase Srs2 disrupts the Rad51 presynaptic filament, *Nature* **423**(6937):305-9.

Kuzminov, A., Schabtach, E., and Stahl, F. W., 1997, Study of plasmid replication in Escherichia coli with a combination of 2D gel electrophoresis and electron microscopy, *J Mol Biol* **268**(1):1-7.

Lee, C. H., and Chung, J. H., 2001, The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation, *J Biol Chem* **276**(32):30537-41.

Lee, S. E., Moore, J. K., Holmes, A., Umezu, K., Kolodner, R. D., and Haber, J. E., 1998, Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage, *Cell* **94**(3):399-409.

Lee, S. E., Pellicioli, A., Malkova, A., Foiani, M., and Haber, J. E., 2001, The Saccharomyces recombination protein Tid1p is required for adaptation from G2/M arrest induced by a double-strand break, *Curr Biol* **11**(13):1053-7.

Lee, S. E., Pellicioli, A., Vaze, M. B., Sugawara, N., Malkova, A., Foiani, M., and Haber, J. E., 2003a, Yeast Rad52 and Rad51 recombination proteins define a second pathway of DNA damage assessment in response to a single double-strand break, *Mol Cell Biol* **23**(23):8913-23.

Lee, S. J., Schwartz, M. F., Duong, J. K., and Stern, D. F., 2003b, Rad53 phosphorylation site clusters are important for Rad53 regulation and signaling, *Mol Cell Biol* **23**(17):6300-14.

Leroy, C., Lee, S. E., Vaze, M. B., Ochsenbien, F., Guerois, R., Haber, J. E., and Marsolier-Kergoat, M. C., 2003, PP2C phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break, *Mol Cell* **11**(3):827-35.

Lisby, M., Barlow, J. H., Burgess, R. C., and Rothstein, R., 2004, Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins, *Cell* **118**(6):699-713.

Lisby, M., Mortensen, U. H., and Rothstein, R., 2003, Colocalization of multiple DNA double-strand breaks at a single Rad52 repair centre, *Nat Cell Biol* **5**(6):572-7.

Llorente, B., and Symington, L. S., 2004, The Mre11 nuclease is not required for 5' to 3' resection at multiple HO-induced double-strand breaks, *Mol Cell Biol* **24**(21):9682-94.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C. S., and Foiani, M., 2001, The DNA replication checkpoint response stabilizes stalled replication forks, *Nature* **412**(6846):557-61.

Lou, Z., Minter-Dykhouse, K., Franco, S., Gostissa, M., Rivera, M. A., Celeste, A., Manis, J. P., van Deursen, J., Nussenzweig, A., Paull, T. T., Alt, F. W., and Chen, J., 2006,

MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals, *Mol Cell* **21**(2):187-200.

Lowndes, N. F., and Murguia, J. R., 2000, Sensing and responding to DNA damage, *Curr Opin Genet Dev* **10**(1):17-25.

Lu, X., Nannenga, B., and Donehower, L. A., 2005, PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints, *Genes Dev* **19**(10):1162-74.

Lucas, I., and Hyrien, O., 2000, Hemicatenanes form upon inhibition of DNA replication, *Nucleic Acids Res* **28**(10):2187-93.

Lucca, C., Vanoli, F., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Haber, J., and Foiani, M., 2004, Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing, *Oncogene* **23**(6):1206-13.

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., and Frohlich, K. U., 2002, A caspase-related protease regulates apoptosis in yeast, *Mol Cell* **9**(4):911-7.

Malkova, A., Naylor, M. L., Yamaguchi, M., Ira, G., and Haber, J. E., 2005, RAD51-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion, *Mol Cell Biol* **25**(3):933-44.

Margolis, S. S., Walsh, S., Weiser, D. C., Yoshida, M., Shenolikar, S., and Kornbluth, S., 2003, PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation, *Embo J* **22**(21):5734-45.

Masumoto, H., Hawke, D., Kobayashi, R., and Verreault, A., 2005, A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response, *Nature* **436**(7048):294-8.

Matsumoto, T., 1997, A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with p34cdc2, *Mol Cell Biol* **17**(2):742-50.

Matsuoka, S., Huang, M., and Elledge, S. J., 1998, Linkage of ATM to cell cycle regulation by the Chk2 protein kinase, *Science* **282**(5395):1893-7.

McGlynn, P., Lloyd, R. G., and Marians, K. J., 2001, Formation of Holliday junctions by regression of nascent DNA in intermediates containing stalled replication forks: RecG stimulates regression even when the DNA is negatively supercoiled, *Proc Natl Acad Sci U S A* **98**(15):8235-40.

Melo, J., and Toczyski, D., 2002, A unified view of the DNA-damage checkpoint, *Curr Opin Cell Biol* **14**(2):237-45.

Melo, J. A., Cohen, J., and Toczyski, D. P., 2001, Two checkpoint complexes are independently recruited to sites of DNA damage in vivo, *Genes Dev* **15**(21):2809-21.

Mochida, S., Esashi, F., Aono, N., Tamai, K., O'Connell, M. J., and Yanagida, M., 2004, Regulation of checkpoint kinases through dynamic interaction with Crb2, *Embo J* **23**(2):418-28.

Morrison, A. J., Highland, J., Krogan, N. J., Arbel-Eden, A., Greenblatt, J. F., Haber, J. E., and Shen, X., 2004, INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair, *Cell* **119**(6):767-75.

Morrison, A. J., Kim, J. A., Person, M. D., Highland, J., Xiao, J., Wehr, T. S., Hensley, S., Bao, Y., Shen, J., Collins, S. R., Weissman, J. S., Delrow, J., Krogan, N. J., Haber, J. E., and Shen, X., 2007, Mec1/Tel1 phosphorylation of the INO80 chromatin remodeling complex influences DNA damage checkpoint responses, *Cell* **130**(3):499-511.

Nakada, D., Hirano, Y., and Sugimoto, K., 2004, Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway, *Mol Cell Biol* **24**(22):10016-25.

Nakada, D., Shimomura, T., Matsumoto, K., and Sugimoto, K., 2003, The ATM-related Tel1 protein of Saccharomyces cerevisiae controls a checkpoint response following phleomycin treatment, *Nucleic Acids Res* **31**(6):1715-24.

Nakamura, T. M., Du, L. L., Redon, C., and Russell, P., 2004, Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast, *Mol Cell Biol* **24**(14):6215-30.

Newlon, C. S., Collins, I., Dershowitz, A., Deshpande, A. M., Greenfeder, S. A., Ong, L. Y., and Theis, J. F., 1993, Analysis of replication origin function on chromosome III of Saccharomyces cerevisiae, *Cold Spring Harb Symp Quant Biol* **58**:415-23.

Nolen, B., Taylor, S., and Ghosh, G., 2004, Regulation of protein kinases; controlling activity through activation segment conformation, *Mol Cell* **15**(5):661-75.

O'Neill, B. M., Szyjka, S. J., Lis, E. T., Bailey, A. O., Yates, J. R., 3rd, Aparicio, O. M., and Romesberg, F. E., 2007, Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage, *Proc Natl Acad Sci U S A* **104**(22):9290-5.

O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C. H., Bobiak, M., Kanai, F., Zhou, B. B., Chung, J. H., and Rathbun, G. A., 2002, Determination of substrate motifs for

human Chk1 and hCds1/Chk2 by the oriented peptide library approach, *J Biol Chem* **277**(18):16102-15.

Osborn, A. J., and Elledge, S. J., 2003, Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53, *Genes Dev* **17**(14):1755-67.

Pan, J., and Chen, R. H., 2004, Spindle checkpoint regulates Cdc20p stability in Saccharomyces cerevisiae, *Genes Dev* **18**(12):1439-51.

Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M., 2000, A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage, *Curr Biol* **10**(15):886-95.

Paulovich, A. G., Toczyski, D. P., and Hartwell, L. H., 1997, When checkpoints fail, *Cell* **88**(3):315-21.

Pellicioli, A., and Foiani, M., 2005, Signal transduction: how rad53 kinase is activated, *Curr Biol* **15**(18):R769-71.

Pellicioli, A., Lee, S. E., Lucca, C., Foiani, M., and Haber, J. E., 2001, Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest, *Mol Cell* **7**(2):293-300.

Pellicioli, A., Lucca, C., Liberi, G., Marini, F., Lopes, M., Plevani, P., Romano, A., Di Fiore, P. P., and Foiani, M., 1999, Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase, *Embo J* **18**(22):6561-72.

Poloumienko, A., Dershowitz, A., De, J., and Newlon, C. S., 2001, Completion of replication map of Saccharomyces cerevisiae chromosome III, *Mol Biol Cell* **12**(11):3317-27.

Redon, C., Pilch, D. R., Rogakou, E. P., Orr, A. H., Lowndes, N. F., and Bonner, W. M., 2003, Yeast histone 2A serine 129 is essential for the efficient repair of checkpoint-blind DNA damage, *EMBO Rep* **4**(7):678-84.

Reid, G. A., and Schatz, G., 1982, Import of proteins into mitochondria. Yeast cells grown in the presence of carbonyl cyanide m-chlorophenylhydrazone accumulate massive amounts of some mitochondrial precursor polypeptides, *J Biol Chem* **257**(21):13056-61.

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S. J., 1999, Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms, *Science* **286**(5442):1166-71.

Sandell, L. L., and Zakian, V. A., 1993, Loss of a yeast telomere: arrest, recovery, and chromosome loss, *Cell* **75**(4):729-39.

Sanders, S. L., Portoso, M., Mata, J., Bahler, J., Allshire, R. C., and Kouzarides, T., 2004, Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage, *Cell* **119**(5):603-14.

Santocanale, C., and Diffley, J. F., 1998, A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication, *Nature* **395**(6702):615-8.

Schulz, V. P., and Zakian, V. A., 1994, The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation, *Cell* **76**(1):145-55.

Schwartz, M. F., Duong, J. K., Sun, Z., Morrow, J. S., Pradhan, D., and Stern, D. F., 2002, Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint, *Mol Cell* **9**(5):1055-65.

Schwartz, M. F., Lee, S. J., Duong, J. K., Eminaga, S., and Stern, D. F., 2003, FHA domain-mediated DNA checkpoint regulation of Rad53, *Cell Cycle* **2**(4):384-96.

Shiloh, Y., 1997, Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart, *Annu Rev Genet* **31**:635-62.

Shiloh, Y., 2003, ATM and related protein kinases: safeguarding genome integrity, *Nat Rev Cancer* **3**(3):155-68.

Shroff, R., Arbel-Eden, A., Pilch, D., Ira, G., Bonner, W. M., Petrini, J. H., Haber, J. E., and Lichten, M., 2004, Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break, *Curr Biol* **14**(19):1703-11.

Smits, V. A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E. A., and Medema, R. H., 2000, Polo-like kinase-1 is a target of the DNA damage checkpoint, *Nat Cell Biol* **2**(9):672-6.

Smolka, M. B., Albuquerque, C. P., Chen, S. H., Schmidt, K. H., Wei, X. X., Kolodner, R. D., and Zhou, H., 2005a, Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine reactive isotope tag, *Mol Cell Proteomics*.

Smolka, M. B., Albuquerque, C. P., Chen, S. H., Schmidt, K. H., Wei, X. X., Kolodner, R. D., and Zhou, H., 2005b, Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine-reactive isotope tag, *Mol Cell Proteomics* **4**(9):1358-69.

Sogo, J. M., Lopes, M., and Foiani, M., 2002, Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects, *Science* **297**(5581):599-602.

Soulier, J., and Lowndes, N. F., 1999, The BRCT domain of the S. cerevisiae checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage, *Curr Biol* **9**(10):551-4.

Strom, L., Lindroos, H. B., Shirahige, K., and Sjogren, C., 2004, Postreplicative recruitment of cohesin to double-strand breaks is required for DNA repair, *Mol Cell* **16**(6):1003-15.

Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson, S. P., 2005, MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks, *Cell* **123**(7):1213-26.

Sun, Z., Fay, D. S., Marini, F., Foiani, M., and Stern, D. F., 1996, Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways, *Genes Dev* **10**(4):395-406.

Sun, Z., Hsiao, J., Fay, D. S., and Stern, D. F., 1998, Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint, *Science* **281**(5374):272-4.

Sweeney, F. D., Yang, F., Chi, A., Shabanowitz, J., Hunt, D. F., and Durocher, D., 2005, Saccharomyces cerevisiae Rad9 acts as a Mec1 adaptor to allow Rad53 activation, *Curr Biol* **15**(15):1364-75.

Szyjka, S. J., Viggiani, C. J., and Aparicio, O. M., 2005, Mrc1 is required for normal progression of replication forks throughout chromatin in S. cerevisiae, *Mol Cell* **19**(5):691-7.

Tamburini, B. A., and Tyler, J. K., 2005, Localized histone acetylation and deacetylation triggered by the homologous recombination pathway of double-strand DNA repair, *Mol Cell Biol* **25**(12):4903-13.

Tanaka, K., Boddy, M. N., Chen, X. B., McGowan, C. H., and Russell, P., 2001, Threonine-11, phosphorylated by Rad3 and atm in vitro, is required for activation of fission yeast checkpoint kinase Cds1, *Mol Cell Biol* **21**(10):3398-404.

Toczyski, D. P., Galgoczy, D. J., and Hartwell, L. H., 1997, CDC5 and CKII control adaptation to the yeast DNA damage checkpoint, *Cell* **90**(6):1097-106.

Tsubouchi, H., and Ogawa, H., 1998, A novel mre11 mutation impairs processing of double-strand breaks of DNA during both mitosis and meiosis, *Mol Cell Biol* **18**(1):260-8.

Ubersax, J. A., Woodbury, E. L., Quang, P. N., Paraz, M., Blethrow, J. D., Shah, K., Shokat, K. M., and Morgan, D. O., 2003, Targets of the cyclin-dependent kinase Cdk1, *Nature* **425**(6960):859-64.

Unal, E., Arbel-Eden, A., Sattler, U., Shroff, R., Lichten, M., Haber, J. E., and Koshland, D., 2004, DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain, *Mol Cell* **16**(6):991-1002.

Usui, T., and Petrini, J. H., 2007, The Saccharomyces cerevisiae 14-3-3 proteins Bmh1 and Bmh2 directly influence the DNA damage-dependent functions of Rad53, *Proc Natl Acad Sci U S A* **104**(8):2797-802.

van Attikum, H., Fritsch, O., and Gasser, S. M., 2007, Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks, *Embo J* **26**(18):4113-25.

van Attikum, H., Fritsch, O., Hohn, B., and Gasser, S. M., 2004, Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair, *Cell* **119**(6):777-88.

Van Hoof, C., Martens, E., Longin, S., Jordens, J., Stevens, I., Janssens, V., and Goris, J., 2005, Specific interactions of PP2A and PP2A-like phosphatases with the yeast PTPA homologues, Ypa1 and Ypa2, *Biochem J* **386**(Pt 1):93-102.

van Leeuwen, F., Gafken, P. R., and Gottschling, D. E., 2002, Dot1p modulates silencing in yeast by methylation of the nucleosome core, *Cell* **109**(6):745-56.

van Vugt, M. A., Bras, A., and Medema, R. H., 2004, Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells, *Mol Cell* **15**(5):799-811.

Vaze, M. B., Pellicioli, A., Lee, S. E., Ira, G., Liberi, G., Arbel-Eden, A., Foiani, M., and Haber, J. E., 2002, Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase, *Mol Cell* **10**(2):373-85.

Veaute, X., Jeusset, J., Soustelle, C., Kowalczykowski, S. C., Le Cam, E., and Fabre, F., 2003, The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments, *Nature* **423**(6937):309-12.

Venclovas, C., and Thelen, M. P., 2000, Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes, *Nucleic Acids Res* **28**(13):2481-93.

Vinella, D., and D'Ari, R., 1995, Overview of controls in the Escherichia coli cell cycle, *Bioessays* **17**(6):527-36.

Weinert, T., 1998, DNA damage checkpoints update: getting molecular, *Curr Opin Genet Dev* **8**(2):185-93.

Weinert, T. A., and Hartwell, L. H., 1988, The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae, *Science* **241**(4863):317-22.

White, C. I., and Haber, J. E., 1990, Intermediates of recombination during mating type switching in Saccharomyces cerevisiae, *Embo J* **9**(3):663-73.

Willson, J., Wilson, S., Warr, N., and Watts, F. Z., 1997, Isolation and characterization of the Schizosaccharomyces pombe rhp9 gene: a gene required for the DNA damage checkpoint but not the replication checkpoint, *Nucleic Acids Res* **25**(11):2138-46.

Wysocki, R., Javaheri, A., Allard, S., Sha, F., Cote, J., and Kron, S. J., 2005, Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9, *Mol Cell Biol* **25**(19):8430-43.

Xu, X., Tsvetkov, L. M., and Stern, D. F., 2002, Chk2 activation and phosphorylation-dependent oligomerization, *Mol Cell Biol* **22**(12):4419-32.

Xu, Y. J., Davenport, M., and Kelly, T. J., 2006, Two-stage mechanism for activation of the DNA replication checkpoint kinase Cds1 in fission yeast, *Genes Dev* **20**(8):990-1003.

Yoo, H. Y., Kumagai, A., Shevchenko, A., and Dunphy, W. G., 2004, Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase, *Cell* **117**(5):575-88.

Zhao, H., Watkins, J. L., and Piwnica-Worms, H., 2002, Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints, *Proc Natl Acad Sci U S A* **99**(23):14795-800.

Zhao, X., Chabes, A., Domkin, V., Thelander, L., and Rothstein, R., 2001, The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage, *Embo J* **20**(13):3544-53.

Zhao, X., Muller, E. G., and Rothstein, R., 1998, A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools, *Mol Cell* **2**(3):329-40.

Zheng, P., Fay, D. S., Burton, J., Xiao, H., Pinkham, J. L., and Stern, D. F., 1993, SPK1 is an essential S-phase-specific gene of Saccharomyces cerevisiae that encodes a nuclear serine/threonine/tyrosine kinase, *Mol Cell Biol* **13**(9):5829-42.

Zhou, B. B., and Elledge, S. J., 2000, The DNA damage response: putting checkpoints in perspective, *Nature* **408**(6811):433-9.

Zou, L., Cortez, D., and Elledge, S. J., 2002, Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin, *Genes Dev* **16**(2):198-208.

Zou, L., and Elledge, S. J., 2003, Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes, *Science* **300**(5625):1542-8.